APPLICATION NOTE AND PROTOCOL

Method	Enzyme Linked Immunosorbent Assay (ELISA)
Detection Range	5 – 640 pM for γH2AX (pS139-H2AX) and 50 – 6400 pM for Total H2AX
Sample Type	18-gauge needle biopsies, xenograft tumor quadrants, and lysates of cultured cells or PBMCs
Sample Size	25 µL lysate/75 µL final well volume

This Research Use Only Document has not been assessed via clinical assay performance standards and is intended for preclinical use. Related SOPs and documentation can be found on the DCTD Biomarkers site at: http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm

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1.0 INTENDED USE

RESEARCH USE ONLY (RUO) Protocol. This SOP is supplied as an RUO protocol for the γH2AX (gamma or phospho-Serine 139 H2AX) and Total H2AX assays for preclinical development laboratory use. Provided here are representative standard curves, performance characteristics, procedures for the assays, preparation of assay controls, detailed material/supply lists, and recommended assay quality control. This RUO SOP does not have an associated formal training program or qualified reagent supply and has not been assessed via clinical performance standards.

2.0 INTRODUCTION

H2AX is a histone H2A variant that constitutes 2–25% of mammalian histone H2A depending on the organism and cell type. Like most other histone proteins, H2AX is composed of a central globular domain, flanked by N-terminal and C-terminal tails which possess sites for a variety of post-translational modifications such as acetylation, biotinylation, phosphorylation, methylation, and ubiquitination. H2AX is structurally similar to other H2A species except for the presence of a unique COOH terminal tail, containing a serine four residues from the C terminus[1]. Upon induction of a DNA double-strand break (DSB), the H2AX omega-4 serine residue becomes rapidly phosphorylated to form gamma-H2AX. Phosphorylated histone H2AX (γH2AX) proves to be an early marker for DNA double-strand breaks, and signal levels directly correlate with the number of breaks formed. Therefore, γH2AX can serve as a pharmacodynamic (PD) marker to measure the chemotoxic effect of potential DNA damaging agents. For this purpose, 96-well ELISAs for quantifying γH2AX and Total H2AX have been developed and are described in this RUO SOP[2-4].

For the γH2AX ELISA, γH2AX is captured from total cell extracts on plates coated with a γH2AX capture monoclonal antibody. The captured protein is then detected using a H2AX polyclonal detection antibody followed by a HRP-conjugate to allow chemiluminescent readout and quantitation of γH2AX levels. The assay for quantifying Total H2AX is based on the same principle using a monoclonal and polyclonal antibody to different epitopes for capture and detection. Using Total H2AX as denominator for γH2AX reporting provides more reliable quantitation of the fraction that is phosphorylated H2AX (γH2AX) and provides a more relevant PD readout for monitoring treatment induced DNA damage.

3.0 CONSIDERATIONS

3.1 Unknown samples are processed following the validated PAR Immunoassay biopsy extraction SOP340520 (http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm)[5].

3.2 These assays were developed with the following specific lot numbers for key reagents. Performance with new lots will need to be assessed by individual laboratories to meet expected performance criteria.

γH2AX Assay

3.2.1 Anti-phospho-H2AX (Ser139) mouse monoclonal antibody, clone JBW301(Millipore, Cat#: 05-636, Lot# DAM1567248) was used at 1:250 dilution (1 µg/µL) for assay development. Other lots used at 1:250 dilutions (1 µg/µL) during development were: Lot# JBC1367868 and Lot# DAM1493341.

3.2.2 Histone H2AX rabbit polyclonal antibody (Abcam, Cat# ab10475, Lot# 778559) was used at 1:850 dilution (1.7 µg/µL) for assay development.

3.2.3 Goat anti-rabbit HRP-conjugated polyclonal antibody (KPL, Cat# 074-15-061, Lot# 101008) was used at 1:1000 dilution (1 µg/µL) for assay development.

3.2.4 γH2AX peptide standard, a lyophilized powder (custom-preparation from Invitrogen, synthetic peptide: AVLLPKKTSATVGPKAPSGGKKATQA[PS]QEY) γH2AX JJ2, Lot# 113011 was used at a standard stock concentration of 20680 pM for assay development.

Total H2AX Assay:

3.2.5 HIST1H2AC monoclonal antibody 4F10 (Novus, Cat#: H00008334-M01, Lot# D8231-4F10) was used at 1:250 dilution (1 µg/µL) for assay development.

3.2.6 Histone H2AX rabbit polyclonal antibody (Abcam, Cat#: ab10475, Lot# 778559) was used at 1:850 dilution (1.7 µg/µL) for assay development.

3.2.7 Goat anti-rabbit HRP-conjugated polyclonal antibody (KPL, Cat#: 074-15-061, Lot# 120504) was used at 1:1000 dilution (1 µg/µL) for assay development.

3.2.8 Total H2AX recombinant protein standard, a lyophilized powder (Axxora, Cat#: ALX-201-176-M005, Lot# L16677) was used at a standard stock concentration of 33000 pM for assay development.

4.0 ASSAY DEVELOPMENT DESCRIPTION

4.1 γH2AX Standard Curve

Following the γH2AX assay protocol described in this RUO SOP, the RLU (relative light unit) values from the synthetic peptide standards were used to generate a standard curve with an Infinite M200 Pro Microplate Reader. An example of a typical γH2AX standard curve is shown below (Figure 1).

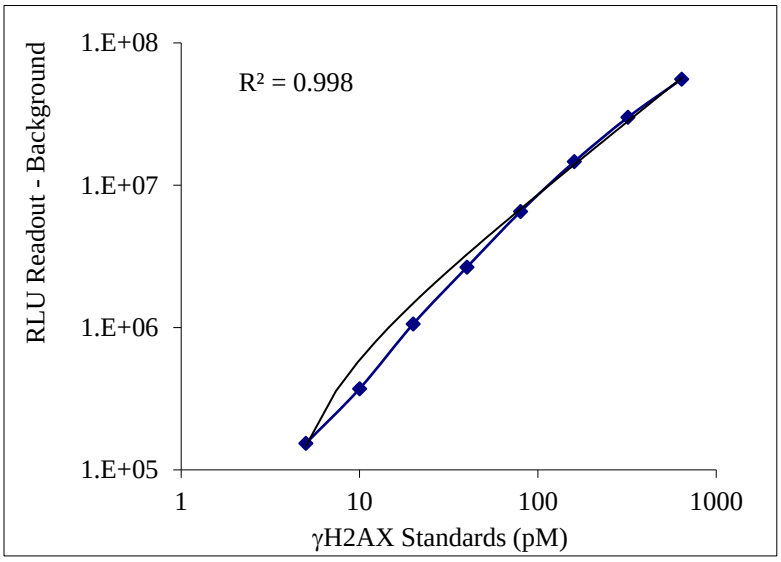

Figure 1. Standard curve for the γH2AX ELISA. This standard curve is for the **purpose of illustration only**, and should not be used to calculate unknowns. Each site should generate its own standard curve and data.

4.2 Total H2AX Standard Curve

Following the Total H2AX assay protocol described in this RUO SOP, the RLU (relative light unit) values from the synthetic peptide standards were used to generate a standard curve with an Infinite M200 Pro Microplate Reader. An example of a typical total H2AX standard curve is shown below (Figure 2).

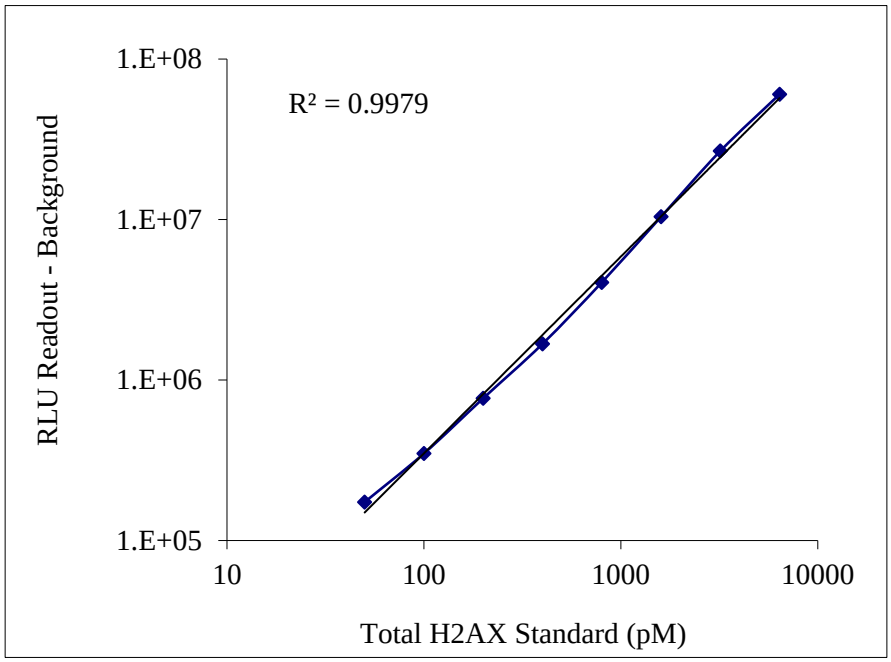

Figure 2. Standard curve for the Total H2AX ELISA. This standard curve is for the **purpose of illustration only**, and should not be used to calculate unknowns. Each site should generate its own standard curve and data.

4.3 γH2AX Assay Performance

The inter-assay performances of the γH2AX assay was evaluated using the defined critical reagent lot numbers in Section 3.2.

Table 1. Inter-assay Precision of the γH2AX assay was measured by 3 runs of γH2AX standards and assay controls. All 3 runs passed the inter-assay acceptance criteria (%CV <25%) with a range from 1 to 12% for the standards and 1.9 to 6.0% for the controls.

γH2AX Standard (pM)	Average RLU (n=3)	SD	%CV
5	992667	122261	12
10	2016850	134112	7
20	4598600	277146	6
40	10021317	467893	5
80	20811817	1459080	7
160	37371983	930881	2
320	58789817	2240098	4
640	79191483	1063448	1
Assay Control	**Average pM (n=3)**	**SD**	**%CV**
Low-C	79.9	1.5	1.9
Mid-C	221.5	7.6	3.4
High-C	558.3	33.4	6.0

4.4 Total H2AX Assay Performance

The intra-assay performances of the Total H2AX were evaluated using the defined critical reagent lot numbers in Section 3.2.

Table 2. Inter-assay precision was measured by 3 runs of total H2AX standards and assay controls. All 3 runs passed the inter-assay acceptance criteria (%CV <25%) with a range from 6 to 21% for the standards and 2.2 to 7.3% for the controls.

Total H2AX Standard (pM)	Average RLU (n=3)	SD	%CV
50	865720	184054	21
100	1063562	168332	16
200	1418546	218046	15
400	2298028	327995	14
800	4611161	370373	8
1600	10482498	851476	8
3200	26055096	1696172	7
6400	59158022	3389425	6

Assay Control	Average pM (n=3)	SD	%CV
Low-C	216.1	15.8	7.3
Mid-C	844.8	49.3	5.8
High-C	2337.0	51.0	2.2

4.5 Tumor lysate controls established during assay development

4.5.1 **γH2AX Readout Ranges** for the prepared lot of MCF7 Control Lysates (Low-C; Mid-C and High-C) used during assay development are provided as a reference below. These values are for the **purpose of illustration only**. Each site should generate its own lot-specific readout ranges.

Tumor Lysate Control Level	Example Acceptable Readout Value (pM)
Low-C	53-82
Mid-C	181-228
High-C	458-583

4.5.2 **Total H2AX Readout Ranges** for the prepared lot of tumor cell lines as Controls (Low-C; Mid-C and High-C) used during assay development are provided as a reference below. These values are for the **purpose of illustration only**. Each site should generate its own lot-specific readout ranges.

Tumor Lysate Control Level	Example Acceptable Readout Value (pM)
Low-C	154 – 275
Mid-C	602 – 1004
High-C	2075 – 2844

4.6 γH2AX /Total H2AX Preclinical Testing

The details of the preclinical testing results were previously presented and published [2-4]. The 96-well plate-based ELISAs for quantifying γH2AX and total H2AX levels have been assessed for in vitro and in vivo applications in irradiation exposure monitoring and in pharmacodynamic evaluation of anti-cancer agents.

In vitro, dose-dependent increases in the ratio of γH2AX to total H2AX were detected after escalating ionizing radiation exposure and concentration-dependent increases after Topoisomerase 1 (Top1) inhibitor exposure. Treating with inhibitors of PARP or ATR alone did not significantly induce γH2AX. Combinations of Top1 inhibitors with PARP or ATR inhibitors led to synergistic induction of DNA damage. Among five ATR inhibitors evaluated in combination with Top1 inhibitors, VE-822 and AZD-6738 were observed to have the highest synergy for γH2AX induction, while NU-6027 showed none. Combinations of CPT-11 (Irinotecan, Camptosar, Campto) with ABT-888, AZD-2281 or MK-4827 showed synergistic induction of γH2AX in A375 xenografts in vivo. Additional testing of human specimens including PBMCs, bone marrow, and tumor biopsies supports the assay's clinical suitability and potential advantages.

To conclude, the quantitative ELISA for measuring both γH2AX and total H2AX is ready for Research Use for monitoring DNA damage induced by chemotherapeutic agents or irradiation.

5.0 MATERIALS AND EQUIPMENT

5.1 γH2AX Key Reagents:

5.1.1 γH2AX peptide standard, lyophilized powder, powder (custom-preparation from Invitrogen, synthetic peptide: AVLLPKKTSATVGPKAPSGGKKATQA[pS]QEY)

5.1.2 Anti-phospho-H2AX (Ser139) mouse monoclonal antibody, clone JBW301 (Millipore, Cat#: 05-636)

5.1.3 Histone H2AX rabbit polyclonal antibody (Abcam, Cat#: ab10475)

5.2 Total H2AX Key Reagents:

5.2.1 Total H2AX recombinant protein standard, lyophilized powder (Axxora, Cat#: ALX-201-176-M005)

5.2.2 HIST1H2AC mouse monoclonal antibody, clone 4F10 (Novus, Cat#: H00008334-M01)

5.2.3 Histone H2AX rabbit polyclonal antibody (Abcam, Cat#: ab10475)

5.3 Tumor Lysate Control (custom preparation prepared to target low, mid and high γH2AX and H2AX ranges)

5.4 Goat anti-rabbit HRP-conjugated polyclonal antibody (KPL, Cat#: 074-15-061). Reconstitute to 1 mg/mL stock solution in HRP Stabilizer (KPL, Cat#: 54-15-01).

5.5 SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific Pierce, Cat#: 37070)

5.6 Acetate plate sealers (Thermo Scientific Pierce, Cat #: 3501)

5.7 Reacti-Bind White Opaque 96-well Plate (Thermo Scientific Pierce, Cat#: 15042)

5.8 Carbonate-bicarbonate buffer capsules, pH 9.6 (e.g., Sigma-Aldrich, Cat#: C3041-50CAP)

5.9 Tween 20 nonionic, aqueous solution, 10% w/v (Roche Applied Science, Cat#: 11332465001)

5.10 20% sodium dodecyl sulfate (SDS; e.g., Sigma-Aldrich, Cat#: 05030-500ML-F)

5.11 10X Phosphate Buffered Saline, pH 7.2 (PBS; e.g., Invitrogen, Cat#: 70013-073)

5.12 SuperBlock (TBS) Blocking Buffer (Thermo Scientific Pierce, Cat#: 37535)

5.13 Albumin, bovine serum (BSA; e.g., Sigma-Aldrich, Cat#: A7030)
5.14 Mouse serum (e.g., Sigma-Aldrich, Cat#: M5905)
5.15 Protamine sulfate salt from salmon (Sigma-Aldrich, Cat#: P4020-5G)
5.16 UltraPure DNase/RNase free distilled water (e.g., Invitrogen, Cat#: 10977-015) or Milli-Q water
5.17 Cell Extraction Buffer (CEB; Invitrogen, Cat#: FNN0011)
5.18 Protease Inhibitor Cocktail (Sigma-Aldrich, Cat#: P-2714 or Roche, Cat#: 11697498001)
5.19 PhosSTOP, phosphatase inhibitor cocktail tablets (Roche, Cat#: 04906837001)
5.20 Pipettors (200-1000 µL, 50-200 µL, and 2-20 µL) and tips
5.21 Multichannel pipettors (50-300 µL, 5-50 µL) and tips
5.22 Reagent reservoirs (Fisher Scientific, Cat#: 21-381-27C)
5.23 1.5-mL Sarstedt tubes (Sarstedt, Cat#: 72.692.005)
5.24 15-mL polypropylene tubes (e.g., Fisher Scientific, Cat#: 14-959-49B)
5.25 50-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352098)
5.26 Ice bucket
5.27 Sorvall Fresco microcentrifuge (Fisher Scientific)
5.28 Vortex Genie 2 (Daigger, Cat#: 3030A)
5.29 Dry, heated incubator able to maintain 37°C ± 3°C
5.30 Dry, heated incubator able to maintain 25°C ± 3°C
5.31 Infinite® 200 or M200Pro Microplate Reader (Tecan US)
5.32 BioTek ELx405 Select Microplate Washer (BioTek Instruments)
5.33 -80°C freezer
5.34 4ºC refrigerator

6.0 EXAMPLE ELISA PLATE CONFIGURATION

6.1 Plate map for γH2AX IA

	1	2	3	4	5	6	7	8	9	10	11	12
A	1X PBS-2% BSA (Assay Buffer) Only					5 pM		1X PBS-2% BSA (Assay Buffer) Only				
B	High-C	S1	S3	S5	S7	10 pM		S9	S11	S13	S15	Low-C
C						20 pM						
D	Mid-C					40 pM						Mid-C
E		S2	S4	S6	S8	80 pM		S10	S12	S14	S16	
F	Low-C					160 pM						High-C
G						320 pM						
H	1X PBS-2% BSA (Assay Buffer) Only					640 pM		1X PBS-2% BSA (Assay Buffer) Only				
	Control Samples	Unknown Samples, Triplicate				γH2AX Peptide Standards, Duplicate		Unknown Samples, Triplicate				Control Samples

S1 through S16 are unknown sample (S) wells in triplicate.

6.2 Plate map for total H2AX IA

	1	2	3	4	5	6	7	8	9	10	11	12
A	1X PBS-2% BSA (Assay Buffer) Only					50 pM		1X PBS-2% BSA (Assay Buffer) Only				
B	High-C	S1	S3	S5	S7	100 pM		S9	S11	S13	S15	Low-C
C						200 pM						
D	Mid-C					400 pM						Mid-C
E		S2	S4	S6	S8	800 pM		S10	S12	S14	S16	
F	Low-C					1600 pM						High-C
G						3200 pM						
H	1X PBS-2% BSA (Assay Buffer) Only					6400 pM		1X PBS-2% BSA (Assay Buffer) Only				
	Control Samples	Unknown Samples, Triplicate				Total H2AX Peptide Standards, Duplicate		Unknown Samples, Triplicate				Control Samples

S1 through S16 are unknown sample (S) wells in triplicate.

6.3 **Optional plate map for running both total H2AX and γH2AX on the same plate.**

	Total H2AX IA						**γH2AX IA**					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	50 pM		1X PBS-2% BSA (Assay Buffer) Only				5 pM		1X PBS-2% BSA (Assay Buffer) Only			
B	100 pM		S1	S3	S5	Mid-C	10 pM		S1a	S3a	S5a	Mid-C
C	200 pM						20 pM					
D	400 pM						40 pM					
E	800 pM		S2	S4	Low-C	High-C	80 pM		S2a	S4a	Low-C	High-C
F	1600 pM						160 pM					
G	3200 pM						320 pM					
H	6400 pM		1X PBS-2% BSA (Assay Buffer) Only				640 pM		1X PBS-2% BSA (Assay Buffer) Only			

Total H2AX Peptide Standards, Duplicate

Unknown and Control Samples, Triplicate

γH2AX Peptide Standards, Duplicate

Unknown and Control Samples, Triplicate

S1 to S5 are unknown sample wells in triplicate for Total H2AX analysis.

S1a to S5a are unknown sample wells in triplicate for γH2AX analysis.

The plate map above can be used for running both total H2AX and γH2AX assays on the same plate if a few samples are used.

7.0 SAMPLE LYSATE PREPARATION

Unknown samples (solid tissue and cells, respectively) should be processed following SOP340520-Biopsy Specimen Processing and SOP340506-PBMC Protein Extraction for the PAR Immunoassay with the following modifications[5]:

- **Landing Page**: http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm
- **SOP340520 LINK:** http://dctd.cancer.gov/ResearchResources/biomarkers/docs/par/SOP340520_Biopsy_Tissue.pdf
- **SOP340506 LINK:** https://dctd.cancer.gov/ResearchResources/biomarkers/docs/par/SOP340506_PBMC_Extraction.pdf

7.1 SOP340520 sample lysis references use of an 18-g needle tumor biopsy. For preclinical sample processing, a similar tissue piece would weigh approximately 10 mg.

7.2 In SOP340520 (tumor tissue) and SOP340506 (PBMC/Cells), add 1 tablet PhosSTOP (Roche Applied Science, Cat#: 04906837001) per 10 mL Cell Extraction Buffer (CEB) for all steps requiring "CEB with Protease Inhibitors (PIs)."

8.0 γH2AX ELISA PLATE SET-UP

8.1 Key Reagents/Supplies

It is recommended to record the lot numbers, stock reagent concentration, and manufacturer's expiration dates for the Key Reagents in the Assay Record (Appendix 1, Section 1). It is advised to follow manufacturer's recommendations for the handling and storage of the reagents/supplies.

8.1.1 **γH2AX Peptide Standard**: Prepared as a 20680 pM stock solution. Aliquot in sufficient volumes for one 96-well plate. NCI has stored the standard successfully at -80°C for > 1 y.

8.1.2 **Tumor Lysate Control**: Lysates prepared from tumor cell lines to target Low, Medium and High γH2AX ranges. NCI has stored the control lysates successfully at -80°C for > 1 y.

See Appendix 3 for details on preparation of tumor lysate controls.

8.1.3 **γH2AX mAb**: Stock solution qualified from the manufacturer. Dilutions for assay performance for specific lot numbers of antibody will need to be determined by the assay site and should be matched to the pAb. Aliquot in sufficient volumes for one 96-well plate. NCI has stored the γH2AX mAb (glycerol containing buffer) successfully at -20°C for > 1 y.

8.1.4 **H2AX pAb**: Stock solution qualified from the manufacturer. Dilutions for assay performance for specific lot numbers of antibody will need to be determined by the assay site and should be matched to the mAb. NCI has stored the H2AX pAb successfully at -80°C for > 1 y.

8.1.5 **Goat Anti-Rabbit HRP-Conjugated pAb**: Prepare a 1 mg/mL stock solution in HRP Stabilizer. Aliquot in sufficient volumes for three 96-well plates. NCI has stored the HRP-Conjugated pAb successfully at 2°C to 8°C for up to 1 y.

8.1.6 **Chemiluminescent Substrate Solutions**: Stock solutions (Peroxide and Pico Luminol/Enhancer Solutions) qualified from the manufacturer. Protect from light during storage and use. NCI has stored the substrate solutions successfully at room temperature at 25°C ± 3°C for up to 1 y.

8.1.7 **Reacti-Bind White Opaque 96-well Plate**: Store at 25°C ± 3°C away from volatile chemicals.

8.2 Plate Map and Buffer Preparation

8.2.1 Based on the number of unknown samples to be analyzed, generate a Plate Map (example, Section 6.1) to define the location and replicates of unknown samples, tumor lysate controls, and γH2AX peptide standards. Samples from a single experiment should be analyzed on one 96-well plate, not split over two plates, to ensure consistent sample handling.

8.2.2 Once the number of wells is known, determine the amount of reagents required for the assay using the Assay Record in Appendix 1. Prepare Wash Buffer and Assay Buffer according to Appendix 1, Section 2A.

IMPORTANT: For all wash and aspiration steps, do not let the wells dry out.

- *Note that it is recommended that both 37°C and 25°C incubation steps for the assay be carried out in fixed-temperature incubators with each 96-well plate placed on a CoolSink thermoconductive plate (pre-warmed for at least 1 hr) during these incubation steps. These thermoconductive plates should be placed horizontally inside the incubator in direct contact with the incubator bottom or shelf and should not be stacked. The assay plate should be placed and carefully centered onto a prewarmed thermoconductive plate inside the incubator for each incubation step.*

8.3 Plate Preparation

8.3.1 Use the calculations in the Assay Record (Appendix 1, Section 3A) to prepare 11 mL γH2AX mAb Coating Solution for the assay. This is sufficient for one 96-well plate (preparing enough for 110 wells). Thaw coating antibody immediately prior to dilution; do not allow to sit for extended periods upon thawing.

8.3.2 If more than one 96-well plate is to be coated, pool the aliquots of coating antibody and then dilute appropriately. This will ensure that all plates are exposed to identical coating antibody. Discard excess diluted antibody.

8.3.3 Add 100 µL of the γH2AX mAb Coating Solution per well using a multichannel pipettor, cover the plate with an acetate sheet, and incubate at 37°C for 2 h. Record the coating antibody incubation conditions in the Assay Record (Appendix1, Section 3B).

8.3.4 Alternatively, the plate can be incubated overnight at 2°C to 8°C.

8.3.5 Following incubation with the γH2AX mAb Coating Solution, aspirate the plate using a plate washer (for the BioTek Plate Washer, use the *Aspirate* program). After aspiration, tap the plate on paper towels to remove any residual liquid.

8.3.6 Add 250 µL of SuperBlock to each well. Cover the plate with an acetate sheet and incubate at 37°C for 1 to 1.5 h. Record the incubation conditions in the Assay Record (Appendix 1, Section 4).

8.3.7 After blocking, move plate to 25°C ± 3°C until washing step (Step 8.7.1).

8.3.8 Alternative, once coated plates have been blocked, they can be stored at 2°C to 8°C for up to one week. It is important to not let wells dry out.

8.4 Unknown Sample Lysate Preparation

8.4.1 Tumor/tissue stock lysates to be analyzed in the assay are initially normalized to 0.2 µg/µL in γH2AX sample diluent (CEB Complete). Tumor stock lysates with a total protein concentration of < 0.2 µg/µL should not be used in the γH2AX Immunoassay.

8.4.1.1 Place unknown samples to be assayed on ice. Record the sample name/IDs and starting lysate concentration in the Assay Record (Appendix 1, Section 5) for each sample to be used.

8.4.1.2 For unknown stock lysates with stock protein concentrations ≥ 0.2 µg/µL, calculate the volume of stock lysate required to prepare 85 µL of a 0.2 µg/µL as follows:

$$\frac{0.2\ \mu g/\mu L}{XX\ \mu g/\mu L\ \text{Stock Lysate}} * 85\ \mu L = XX\ \mu L\ \text{Vol. Stock Lysate}$$

8.4.1.3 Record the volumes stock lysate, γH2AX sample diluent (CEB Complete) and concentration of working dilutions of the lysate in the Assay record (Appendix 1, Section 5).

8.4.1.4 Do not pipette less than 5 µL of the stock lysate. If the calculations above yield volumes of stock lysate less than 5 µL, prepare sufficient volume of a 1:2 to 1:5 pre-dilution of the lysate before proceeding.

8.4.1.5 In a labeled 1.5 mL tube, add sufficient γH2AX sample diluent (CEB Complete) to the calculated volume of stock lysate needed to bring total volume to 85 µL. Keep the working lysate on ice.

8.4.1.6 Flash freeze remaining stock lysate in liquid nitrogen or dry/ice ethanol and return to -80˚C freezer. As with all lysates, freeze/thaw cycles should be minimized.

8.4.2 From the normalized working tumor/tissue lysates of 0.2 µg/µL, three final dilutions are prepared in order to analyze the lysates at 2, 1 and 0.5 µg/well in γH2AX sample diluent (CEB Complete).

8.4.2.1 Perform the following calculation to calculate the volume of working lysate needed to prepare 3 different lysate dilutions (2, 1, or 0.5 µg/well).

$$\frac{2,\ 1\ \text{or}\ 0.5\ \mu g/\text{well}}{0.2\ \mu g/\mu L\ \text{Working Lysate}} * 4\ \text{wells} = 40, 20\ \text{or}\ 10\ \mu L\ \text{Working Lysate}$$

8.4.2.2 For each Diluted Lysate, γH2AX sample diluent (CEB Complete) should be used to bring the total volume to 100 µL. This is sufficient volume to run each dilution in triplicate (plus 1 extra well).

8.4.2.3 Record the Sample ID, volume Working Lysate and γH2AX sample diluent (CEB Complete) used to prepare each Diluted Tumor Lysate in the Assay Record (Appendix 1, Section 6A).

8.4.2.4 Clearly label 1.5 mL tubes with the sample number (e.g., S1, S2), add sufficient volume γH2AX sample diluent (CEB Complete) to the calculated volume of working lysate to bring the total volume to 100 µL.

8.4.2.5 Keep the Diluted Lysates on ice until use. Discard remaining unused Working Lysate.

8.4.3 For preparation of PBMC/Cell Lysates samples:

8.4.3.1 Stock lysates for PBMCs/Cells (1 x 10^7 cells/mL) are prepared according to Section 7.0 and Appendix 3.

8.4.3.2 Place 100 µL of the stock lysate into a 1.5 mL tube labeled with the sample number (e.g., S1, S2). No other sample preparation is necessary; this is enough for triplicate well preparation (+1 well extra).

8.4.3.3 Record the stock lysate concentration in cells/mL, the volume of stock PBMC/Cell lysate set aside for each sample in the Assay Record (Appendix 1, Section 6A).

8.4.3.4 Lysates will be diluted an additional 3-fold once loaded into the 96-well plate yielding a relative load of 2.5 x 10^5 cells/well. This relative load will provide quantitative values in the range of the assay for most PBMC and cellular lysates. It may be necessary to pre-dilute lysates prepared from certain cell lines with high levels of γH2AX. In the event that a cell line provides quantitative values above the range of the assay (>ULQ) the samples should be prediluted in CEB Complete prior to analysis.

8.4.3.5 Keep the PBMC/Cell lysates aliquoted for use in the assay on ice until use. Flash freeze the remaining stock lysate in liquid nitrogen or dry ice/ethanol bath and return to -80˚C freezer. As with all lysates, freeze/thaw cycles should be minimized.

8.5 Preparation of γH2AX peptide standards (run in duplicate)

8.5.1 For one 96-well plate, retrieve a γH2AX peptide standard stock tube (20680 pM) from the -80°C freezer and thaw on ice. Vortex and mix by inverting 5-8 times before use. Label eight 1.5-mL tubes, numbered 1 through 8, for the γH2AX peptide standards. Prepare a 9th tube as the blank.

8.5.2 Prepare the γH2AX peptide standards by serial dilution as outlined in the Assay Record (Appendix 1, Section 6B) with final concentrations ranging from 1920 to 15 pM in Assay Buffer (1X PBS-2% BSA).

8.5.3 Standards will be diluted an additional 3-fold when added to the 96-well plate to generate a reference curve ranging from 640 to 5 pM γH2AX peptide standard. Keep standards on ice until use. Only make enough standards for the assay and discard any excess.

8.6 Control Lysates (run in duplicate)

8.6.1 For one 96-well plate, retrieve one of each High-C, Mid-C and Low-C tumor lysate control vials from the -80°C freezer and thaw on ice. Controls are prepared at a concentration ready for use in the assay and no further dilution is required. Vortex and mix by inverting 5-8 times before use. See Appendix 3 for more information on preparation of controls.

8.6.2 Keep controls on ice until use. Controls will be diluted 3-fold with Assay Buffer (1X PBS-2% BSA) once loaded into the 96-well plate. Only thaw enough of the controls for the assay and discard any excess.

8.6.3 Note the Control Lot Numbers in Appendix 1, Section 6C.

8.7 γH2AX Protein Capture

8.7.1 Following incubation with SuperBlock (Step 8.3.6), the plates are aspirated and washed once with 350 µL of Wash Buffer (1X PBS-0.1% Tween) using a plate washer.

For the BioTek Microplate Washer, the settings are:

METHOD	
Number of Cycles:	**1**
Soak/Shake:	**Yes**
Soak Time:	**5 Sec**
Shake before soak:	**No**
Prime after soak:	**No**
DISPENSE	
Dispense Volume:	**350 µL/well**
Dispense Flow Rate:	**06**
Dispense Height:	**120 (15.240 mm)**
Horizontal DISP POS:	**00 (0.000 mm)**
Bottom Wash First:	**No**
Prime Before Start:	**No**
ASPIRATE	
Aspirate Height:	**031 (3.937 mm)***
Horizontal ASPR POS:	**-20 (-0.914 mm)***
Aspiration Rate:	**05 (6.4 mm/sec)**
Aspirate Delay:	**1000 MSec**
Crosswise ASPIR:	**No**
Final Aspiration:	**Yes**
Final Aspirate Delay:	**1000 MSec**

*Recommended initial setting, optimize Aspirate Height and Horizontal ASPR POS to allow for complete aspiration of an individual unit following manufacturer's recommendations.

8.7.2 After the wash, tap the plate on paper towels to remove residual buffer. Proceed immediately to the next step; do not allow the plate to dry out.

8.7.3 Immediately, add 50 µL of Assay Buffer (1X PBS-2% BSA) to each well using a multichannel pipettor. Each well will hold a final volume of 75 µL after sample addition.

8.7.4 Use the Plate Map Design (Section 6.1) and the Sample Calculation Table (Appendix 1, Section 6A) as a guide to set up the 96-well plate for incubation with unknown samples, γH2AX peptide standards (Appendix 1, Section 6B), and tumor cell controls (Appendix 1, Section 6C and Appendix 3). Pipette reagents in the following order; **do not deviate** from order of addition:

Order	Sample/Reagent and Volume
1	25 µL of specified concentrations of γH2AX peptide standards into designated duplicate wells. Load the lowest concentration first.
2	25 µL of each unknown sample into designated triplicate wells.
3	25 µL each of assay controls (Low-C, Mid-C, and High-C) into both sets of designated duplicate wells.
4	25 µL of additional Assay Buffer (1X PBS-2% BSA) into each of the Background wells.

8.7.5 Cover the plate with an acetate sheet and incubate at 2°C to 8°C for 18 ± 2 h. Record the date, start time, and incubation temperature in the Assay Record (Appendix 1, Section 7).

8.8 γH2AX Detection (next day)

Go to Section 10.0 for the Detection Method which is the same for both the γH2AX and Total H2AX assays**.**

9.0 Total H2AX ELISA PLATE SET UP

9.1 Key Reagents/Supplies

It is recommended to record the lot numbers, stock reagent concentration, and manufacturer's expiration dates for the Key Reagents in the Assay Record (Appendix 2, Section 1). It is advised to follow manufacturer's recommendations for the handling and storage of the reagents/supplies.

9.1.1 **Total H2AX Peptide Standard**: Prepared as a 33000 pM stock solution. Aliquot in sufficient volumes for one 96-well plate. NCI has stored successfully at -80°C for > 1 y.

9.1.2 **Tumor Lysate Control**: Lysates from tumor cell lines prepared to target Low, Medium and High Total H2AX ranges. NCI has stored successfully at -80°C for > 1 y.

See Appendix 3 for details on preparation of tumor cell line lysate controls.

9.1.3 **Total H2AX mAb**: Stock solution qualified from the manufacturer. Dilutions needed for assay performance of specific lot numbers of antibody will need to be determined by the assay site and should be matched to the pAb. NCI has stored successfully at -80°C for > 1 y.

9.1.4 **H2AX pAb**: Stock solution qualified from the manufacturer. Dilutions needed for assay performance of specific lot numbers of antibody will need to be determined by the assay site and should be matched to the mAb. Aliquot in sufficient volumes for one 96-well plate. NCI has stored successfully at -80°C for > 1 y.

9.1.5 **Goat Anti-Rabbit HRP-Conjugated pAb**: Prepare a 1 mg/mL stock solution in HRP Stabilizer. Aliquot in sufficient volumes for three 96-well plates. NCI has stored successfully at 2°C to 8°C for up to 1 y.

9.1.6 **Chemiluminescent Substrate Solutions**: Stock solutions (Peroxide and Pico Luminol/Enhancer Solutions) qualified from the manufacturer. Protect from light during storage and use. NCI has stored successfully at 25°C ± 3°C for up to 1 y.

9.1.7 **Reacti-Bind White Opaque 96-well Plate**: Store at 25°C ± 3°C away from volatile chemicals.

9.2 Plate Map and Buffer Preparation

9.2.1 Based on the number of unknown samples to be analyzed, generate a Plate Map (Section 6.2) to define the location and replicates of unknown samples, tumor lysate controls, and Total H2AX peptide standards. Samples from a single experiment should be analyzed on one 96-well plate, not split over two, to ensure consistent sample handling.

9.2.2 Once the number of wells is known, determine the amount of reagents required for the assay using the Assay Record in Appendix 2.

IMPORTANT: For all wash and aspiration steps, do not let the wells dry out.

- *Note that it is recommended that both 37°C and 25°C incubation steps for the assay be carried out in fixed-temperature incubators with each 96-well plate placed on a CoolSink thermoconductive plate (pre-warmed for at least 1 hr) during these incubation steps. These thermoconductive plates should be placed horizontally inside the incubator in direct contact with the incubator bottom or shelf and should not be stacked. The assay plate should be placed and carefully centered onto a prewarmed thermoconductive plate inside the incubator for each incubation step.*

9.3 Plate Preparation

9.3.1 Use the calculations in the Assay Record (Appendix 2, Section 3A) to prepare 11 mL Total H2AX mAb Coating Solution for the assay. This is sufficient for one 96-well plate (preparing enough for 110 wells). Thaw coating antibody immediately prior to dilution; do not allow to sit for extended periods upon thawing.

9.3.2 If more than one 96-well plate is to be coated, pool the coating antibody aliquots and then dilute appropriately. This will ensure that all plates are exposed to identical coating antibody. Discard excess diluted antibody.

9.3.3 Add 100 µL of the Total H2AX mAb Coating Solution per well using a multichannel pipettor, cover the plate with an acetate sheet, and incubate at 37°C for 2 h. Record the coating antibody incubation conditions in the Assay Record (Appendix 2, Section 3B).

9.3.4 Alternatively, the plate can be incubated overnight at 2°C to 8°C.

9.3.5 Following incubation with the Total H2AX mAb Coating Solution, aspirate the plate using a plate washer (for the BioTek Plate Washer, use the *Aspirate* program). After aspiration, tap the plate on paper towels to remove any residual liquid.

9.3.6 Add 250 µL of SuperBlock to each well. Cover the plate with an acetate sheet and incubate at 37°C for 1 to 1.5 h. Record the incubation conditions in the Assay Record (Appendix 2, Section 4).

9.3.7 After blocking, move plate to 25°C ± 3°C until washing step (Step 9.5.1).

9.3.8 Alternatively, once coated plates have been blocked they can be stored at 2°C to 8°C for up to one week. It is important to not let wells dry out.

9.4 Unknown Sample Lysate Preparation

9.4.1 Tumor/tissue stock lysates with stock protein concentrations > 0.75 µg/µL are initially prediluted to 0.75 µg/µL. For stock lysates with protein concentrations between 0.2 and 0.75 µg/µL, no predilution is required and the stock lysates are used directly to prepare the final assay lysate preparations as described below. Unknown sample lysates with a total protein concentration of < 0.2 µg/µL should not be used in the Total H2AX Immunoassay.

9.4.2 For unknown stock tumor/tissue lysates with stock protein concentrations > 0.75 µg/µL:

9.4.2.1 Place unknown samples to be assayed on ice. Record the sample name/IDs and starting lysate concentration in the Assay Record (Appendix 2, Section 5) for each sample to be used.

9.4.2.2 Calculate the volume of stock lysate required to prepare 10 µL of a 0.75 µg/µL Working Lysate as follows:

$$\frac{0.75\ \mu g/\mu L}{XX\ \mu g/\mu L\ \text{Stock Lysate}} * 10\ \mu L = XX\ \mu L\ \text{Vol. Stock Lysate}$$

9.4.2.3 Record the stock lysate concentration, working lysate concentration, volume stock lysate and volume H2AX sample diluent (CEB Complete) in the Assay Record (Appendix 2, Section 5).

9.4.2.4 Do not pipette less than 5 µL of the stock lysate. If the calculations above yield volumes of stock lysate less than 5 µL, prepare sufficient volume of a 1:2 to 1:5 pre-dilution of the lysate before proceeding.

9.4.2.5 In a labeled 1.5 mL tube, add sufficient Total H2AX Sample Diluent (CEB Complete) to the calculated volume of stock lysate needed to bring the total volume to 10 µL.

9.4.2.6 Add 65 µL of Assay Buffer (1X PBS-2% BSA) to generate 75 µL of 0.1 µg/µL Diluted Working Lysate. Record the volume prediluted lysate (10 µL), diluted working lysate concentration (0.1 µg/µL) and volume Assay Buffer (65 µL) in the Assay Record (Appendix 2, Section 5).

9.4.2.7 Keep the diluted working lysate on ice. Flash freeze remaining stock lysate in liquid nitrogen or dry ice/ethanol and return to -80˚C freezer. As with all lysates, freeze/thaw cycles should be minimized.

9.4.3 For unknown stock lysates with stock protein concentrations between 0.2 and 0.75 µg/µL, no normalization is needed and stock lysate are directly diluted with Assay Buffer (1X PBS-2% BSA) for the Diluted Working Lysate of 0.1 µg/µL.

9.4.3.1 Place unknown samples to be assayed on ice. Record the sample name/IDs and starting lysate concentration in the Assay Record (Appendix 2, Section 5) for each sample to be used.

9.4.3.2 Calculate the volume of stock lysate required to prepare 75 µL of a 0.1 µg/µL Working Lysate as follows:

$$\frac{0.1\ \mu g/\mu L}{XX\ \mu g/\mu L\ \text{Stock Lysate}} * 75\ \mu L = XX\ \mu L\ \text{Vol. Stock Lysate}$$

9.4.3.3 Record the stock lysate concentration, working lysate concentration, volume stock lysate and volume Assay Buffer (1X PBS-2% BSA) in the Assay Record (Appendix 2, Section 5).

9.4.3.4 In a labeled 1.5 mL tube, add sufficient volume Assay Buffer (1X PBS—2% BSA) to the calculated volume of stock lysate needed to bring the total volume to 75 µL.

9.4.3.5 Keep the diluted working lysate on ice. Flash freeze remaining stock lysate in liquid nitrogen or dry ice/ethanol and return to -80˚C freezer. As with all lysates, freeze/thaw cycles should be minimized.

9.4.4 From the normalized working tumor/tissue lysate of 0.1 µg/µL, three final dilutions are prepared in order to analyze the lysates at 0.5, 0.375 and 0.25 µg/well.

9.4.4.1 Perform the following calculation to calculate the volume of normalized working lysate needed to analyze the lysates at 0.5, 0.375 and 0.25 µg/well.

$$\frac{0.5,\ 0.375\ \text{or}\ 0.25\ \mu g/\text{well}}{0.1\ \mu g/\mu L\ \text{Working Lysate}} * 4\ \text{wells} = 20,\ 15\ \text{or}\ 10\ \mu L\ \text{Working Lysate}$$

9.4.4.2 For each diluted working lysate, Assay Buffer (1X PBS-2% BSA) should be used to bring the total volume to 100 µL. This is sufficient volume to run each dilution in triplicate (+1 well extra).

9.4.4.3 Record the Specimen ID, Working Lysate Concentration, Volume Working Lysate and Assay Buffer used to prepare each final diluted lysate in the Assay Record (Appendix 2, Section 6A).

9.4.4.4 Clearly label 1.5 mL tubes with the sample number (e.g., S1, S2), add sufficient volume Assay Buffer to the calculated volume of Working Lysate to bring the total volume to 100 µL.

9.4.4.5 Keep the Diluted Lysates on ice until use. Discard remaining unused Working Lysate.

9.4.5 For preparation of PBMC/Cell Lysate samples:

9.4.5.1 Stock lysates for PBMCs/Cells (1 x 10^7 cells/mL) are prepared according to Section 7.0 and Appendix 3.

9.4.5.2 PBMC/Cell diluted lysates are prepared by adding 7.5 µL of the stock lysate into 117.5 µL of Assay Buffer (1X PBS-2% BSA) to a final concentration of 6 x 10^5 cells/mL in 125 µL for the Total H2AX assay. Record the dilution information in the Sample Calculation Table (Appendix 2, Section 6A).

9.4.5.3 Clearly label all tubes with the sample number (e.g., S7, S8) and add 7.5 µL of the stock lysate into 117.5 µL of 1X PBS-2% BSA for each sample.

9.4.5.4 Lysates will be diluted an additional 3-fold once loaded into the 96-well plate with various assay reagents yielding a relative load of 1.5 x 10^4 cells/well.

9.4.5.5 Keep the diluted PBMC/Cell lysates on ice until use. Flash freeze the remaining stock lysate in liquid nitrogen or dry ice/ethanol bath and return to -80˚C freezer. As with all lysates, freeze/thaw cycles should be minimized.

9.4.6 Preparation of Total H2AX Recombinant Standards (run in duplicate)

9.4.6.1 For one 96-well plate, retrieve one Total H2AX recombinant standard working stock solution tube (33000 pM) from the -80°C freezer and thaw on ice. Vortex and mix by inverting 5-8 times before use. Label eight 1.5 mL tubes, numbered 1 through 8, for the Total H2AX recombinant standards. Prepare a 9th tube of only Assay Buffer (1X PBS-2% BSA) to load into the background wells.

9.4.6.2 Prepare the Total H2AX recombinant standards by serial dilution as outlined in the Assay Record (Appendix 2, Section 6B) with concentrations ranging from 19200 to 150 pM in Assay Buffer (1X PBS-2% BSA).

9.4.6.3 Standards will be diluted an additional 3-fold when added to the 96-well plate to generate a reference curve ranging from 6400 to 50 pM Total H2AX recombinant standard.

9.4.6.4 Keep standards on ice until use. Only make enough standards for the assay and discard any excess

9.4.7 Preparation of Tumor Cell Lysate Controls (run in duplicate)

9.4.7.1 For one 96-well plate, retrieve one of each High-C, Mid-C and Low-C tumor lysate control vial from the -80°C freezer and thaw on ice. Controls are provided at a concentration ready for use in the assay and no further dilution is required. Vortex and mix by inverting 5-8 times before use (Appendix 2, Section 6C). See Appendix 3 for more information on preparation of controls.

9.4.7.2 Keep controls on ice until use. Controls will be diluted 3-fold with Assay Buffer (1X PBS-2% BSA) once loaded into the 96-well plate.

9.4.7.3 Note the Control Lot Numbers on the Assay Record (Appendix 2, Section 6C).

9.5 Total H2AX Protein Capture

9.5.1 Following incubation with SuperBlock (SOP Step 9.3.6), the plates are aspirated and washed once with 350 µL of Wash Buffer (1X PBS-0.1% Tween) using a plate washer.

For the BioTek Microplate Washer, the settings are:

METHOD	
Number of Cycles:	**1**
Soak/Shake:	**Yes**
Soak Time:	**5 Sec**
Shake before soak:	**No**
Prime after soak:	**No**
DISPENSE	
Dispense Volume:	**350 µL/well**
Dispense Flow Rate:	**06**
Dispense Height:	**120 (15.240 mm)**
Horizontal DISP POS:	**00 (0.000 mm)**
Bottom Wash First:	**No**
Prime Before Start:	**No**
ASPIRATE	
Aspirate Height:	**031 (3.937 mm)***
Horizontal ASPR POS:	**-20 (-0.914 mm)***
Aspiration Rate:	**05 (6.4 mm/sec)**
Aspirate Delay:	**1000 MSec**
Crosswise ASPIR:	**No**
Final Aspiration:	**Yes**
Final Aspirate Delay:	**1000 MSec**

*Recommended initial setting, optimize Aspirate Height and Horizontal ASPR POS to optimize complete aspiration for an individual unit following manufacturer's recommendations.

9.5.2 After the wash, tap the plate on paper towels to remove residual buffer. Proceed immediately to the next step; do not allow the plate to dry out.

9.5.3 Immediately, add 50 µL of Assay Buffer (1X PBS-2% BSA) containing 100 ng/well of Protamine to each well using a multichannel pipettor. Each well will hold a final volume of 75 µL after sample addition.

9.5.4 Use the Plate Map Design (Section 6.2) and the Sample Calculation Table (Appendix 2, Section 6A) as a guide to set up the 96-well plate for incubation with samples, Total H2AX standards (Appendix 2, Section 6B), and assay controls (Appendix 2, Section 6C and Appendix 3). Pipette reagents/lysates in the following order; **do not deviate** from order of addition:

Order	Sample/Reagent and Volume
1	25 µL of specified concentrations of Total H2AX standards into designated duplicate wells. Load the lowest concentration first.
2	25 µL of each unknown sample into designated triplicate wells.
3	25 µL each of assay control (Low-C, Mid-C, and High-C) into both sets of designated duplicate wells.
4	25 µL of additional 1X PBS-2% BSA into each of the Background wells.

9.5.5 Cover the plate with an acetate sheet and incubate at 2°C to 8°C for 18 ± 2 h. Record the date, start time, and incubation temperature in the Assay Record (Appendix 2, Section 7).

10.0 DETECTION FOR BOTH γH2AX AND TOTAL H2AX ASSAYS (NEXT DAY)

10.1 Detection Antibody Incubation

10.1.1 Prepare a sufficient amount of the rabbit detection pAb 15 min before washing the plate(s) after the capture incubation for the γH2AX and/or Total H2AX assays.

10.1.2 Using the calculations in Appendices 1 and/or 2, Sections 8A, to prepare the rabbit detection pAb working solution in Assay Buffer (1X PBS-2% BSA). Be sure to record the lot number of mouse serum used.

10.1.3 Allow the prepared rabbit detection pAb to incubate for 15 min at 25°C ± 3°C and record this incubation in Appendices 1 and/or 2, Section 8A.

10.1.4 After the 18-h incubation of the coated plates with samples is complete, aspirate and wash the wells 4 times with 350 µL of Wash Buffer (1X PBS-0.1% Tween) using the same wash program as Step 8.7.1, except run for **4 cycles**. Record the date and time samples were removed from the wells in the Assay Record (Appendices 1 and/or 2, Section 7).

10.1.5 After the wash, tap the plate on paper towels to remove residual Wash Buffer. Proceed immediately to the next step; do not allow the plate to dry out.

10.1.6 Add 100 µL of the rabbit detection pAb working solution per well using a multichannel pipettor, cover the plate with an acetate sheet, and incubate for 2 to 2.5 h at 25°C ± 3°C. Discard residual working solution and record the incubation conditions in the Assay Record (Appendices 1 and/or 2, Section 8B).

10.1.7 15 min before the incubation with the rabbit detection pAb is complete, prepare a sufficient amount of HRP conjugate for the assay. Using the calculations in Appendices 1 and/or 2, Sections 9A, prepare the HRP conjugate working solution in Assay Buffer.

10.1.8 Allow the prepared HRP conjugate to incubate in the dark at 25°C ± 3°C for 15 min and record the incubation conditions in the Assay Record (Appendices 1 and/or 2, Section 9A).

10.1.9 After the 2 to 2.5 h incubation with the rabbit detection pAb is complete, aspirate and wash the wells 4 times with 350 µL of Wash Buffer (1X PBS-0.1% Tween) using the same wash program as SOP Step 8.7.1, except run for **4 cycles**. Tap plate on paper towels to remove residual liquid and proceed immediately to the next step.

10.1.10 Add 100 µL of the HRP conjugate working solution per well using a multichannel pipettor. Cover the plate with an acetate sheet and incubate in the dark for 1 to 1.5 h at 25°C ± 3°C. Discard residual working solution and record the incubation conditions in the Assay Record (Appendices 1 and/or 2, Section 9B).

10.2 **Signal Detection**

10.2.1 Turn on the Tecan Infinite Plate Reader at least 30 min before use. For luminescence optical density readings, the plate reader should be set to the following reading parameters:

Shaking duration:	**5 sec**
Mode:	**linear**
Amplitude:	**1 mm**
Attenuation:	**OD1**
Integration Time:	**100 ms**

10.2.2 Just before the HRP conjugate incubation is finished, prepare SuperSignal ELISA Pico Chemiluminescent Substrate Solution as outlined in Appendices 1 and/or 2, Section 10A, being sure to note the time of preparation. This must be made up immediately before use, kept in the dark, and at a sufficient volume for the assay.

10.2.3 After the 1 to 1.5 h HRP conjugate incubation is complete, aspirate and wash the wells 4 times with 350 µL of Wash Buffer (1X PBS-0.1% Tween) using the same wash program as SOP Step 8.7.1, except run for **4 cycles**. Tap plate on a paper towel to remove excess buffer and proceed immediately to the next step.

10.2.4 Add 100 µL of the freshly made Substrate Solution per well with a multichannel pipettor and avoid bright light. Record the time of addition to wells (Appendices 1 and/or 2, Section 10B).

10.2.5 The first chemiluminescence reading should be within 2 min of substrate addition. Record the time of the initial relative light unit (RLU) reading in the Assay Record (Appendices 1 and/or 2, Section 10B).

10.2.5.1 Refer to Sections 4.3 or 4.4 for examples of expected RLU readout ranges for standards and tumor cell lysate controls for the γH2AX and Total H2AX assays, respectively.

10.2.5.2 If the signal is too high from the initial reading, wait 5 min and read the plate again at the same instrument setting. Continue reading until the RLU signal is on scale.

10.2.5.3 Record time the final RLU reading is taken in Appendices 1 and/or 2, Section 10B.

10.2.6 Save the resulting readings.

10.2.7 The equation for the line (y=mx+b) of the standard curve can be used to convert RLU readings of the unknowns to γH2AX and Total H2AX readings in pM.

10.3 Review and finalize the Assay Records (Appendices 1 and/or 2). Document ANY and ALL deviations from this SOP in the Assay Record (Appendices 1 and/or 2, Section 11).

11.0 QUALITY CONTROL RECOMMENDATIONS FOR γH2AX/TOTAL H2AX ASSAYS

11.1 Background Well QC

11.1.1 From the "Plate Map Design" identify the background wells that are to be used in QC determination. A total of 14 wells are used for background determination; the 4 corner wells and 2 adjacent to the high standard are not used for background level calculation.

11.1.2 A ± 2 SD criterion is applied to the initial 14-well dataset to identify outliers.

11.1.3 If a background well RLU value is ≥ 2 SD from the mean, delete that value from the background data set.

11.1.4 Once all wells that were ≥ 2 SD from the initial background data set mean have been deleted, the %CV for the background wells must be < 20%.

11.1.5 If the %CV for the background wells is < 20%, the assay passes QC, proceed to Step 11.2.

11.1.6 If the %CV is ≥ 20%, the Assay Fails QC, do not continue with the analysis. State in the Assay Record (Appendices 1 and/or 2, Section 11) the reason for assay failure. Rerun the assay with fresh reagents.

11.2 Standard Curve QC

11.2.1 Low Standard QC and LLQ Assignment for γH2AX Assay:

11.2.1.1 In order to use the 5 to 10 pM range of the standard curve, the mean RLU readout of the 5 pM standard must be ≥ 3 SD above the mean RLU readout of the background; this value is referred to as the LLQ-RLU.

11.2.1.2 If the 5 pM standard fails, then the mean RLU readout of the 10 pM standard must be ≥ 3 SD above the mean RLU readout of the background.

11.2.1.3 If the 10 pM standard also fails, the Assay Fails QC.

11.2.1.4 The lowest passing standard is assigned as the LLQ (pM) for the assay.

11.2.2 Signal-to-background (S/B) ratio QC and ULQ Assignment, γH2AX:

11.2.2.1 The ratio for the lowest passing standard (5 or 10 pM) RLU readout to the mean RLU readout of the background must be ≥ 1.1. If not, the Assay Fails QC.

11.2.2.2 The ratio of the highest standard RLU readout (640 pM) to the mean RLU of the background must be ≥ 15. If not, the Assay Fails QC.

11.2.2.3 If the high standard passes QC, it is assigned as the ULQ (pM) for the assay.

11.2.3 Low Standard QC and LLQ Assignment, Total H2AX Assay:

11.2.3.1 In order to use the 50 to 100 pM range of the standard curve, the mean RLU readout of the 50 pM standard must be ≥ 3 SD above the mean RLU readout of the background; this value is referred to as the LLQ-RLU.

11.2.3.2 If the 50 pM standard fails, then the mean RLU readout of the 100 pM standard must be ≥ 3 SD above the mean RLU readout of the background.

11.2.3.3 If the 100 pM standard also fails, the Assay Fails QC.

11.2.3.4 The lowest passing standard is assigned as the LLQ (pM) for the assay.

11.2.4 Signal-to-background (S/B) ratio QC and ULQ Assignment, Total H2AX Assay:

11.2.4.1 The ratio for the lowest passing standard (50 or 100 pM) RLU readout to the mean RLU readout of the background must be ≥ 1.1. If not, the Assay Fails QC.

11.2.4.2 The ratio of the highest standard RLU readout (6400 pM) to the mean RLU of the background must be ≥ 15. If not, the Assay Fails QC.

11.2.4.3 If the high standard passes QC, it is assigned as the ULQ (pM) for the assay.

11.3 Control Samples

11.3.1 The QC determination for the control samples should have the following criteria:

11.3.1.1 At least one control at each level (Low-, Mid-, and High-C) must have a CV of < 20% for the replicate wells.

11.3.1.2 At least one control at each level and at least 4 of 6 controls overall must fall within the defined γH2AX or Total H2AX pM range determined for the specific lot of critical reagent.

11.3.2 If any of these criteria are not met, the Assay Fails QC. State in the Assay Record (Appendices 1 and/or 2, Section 11) the reason for assay failure. Rerun the assay with fresh reagents.

11.4 Unknown Sample Replicate QC and LLQ/ULQ QC

11.4.1 Triplicate repeats for each sample must have a CV < 20%.

11.4.2 Review the average γH2AX or Total H2AX levels and identify any values that are < LLQ or > ULQ.

11.4.2.1 If a sample is > ULQ and there is sufficient sample volume, it can be re-run with fresh reagents at a lower protein or cell number load/well. If a sample is < LLQ and there is sufficient sample volume, it can be re-run at a higher protein or cell number load/well.

12.0 REFERENCES

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2. Ji J, Zhang Y, Kinders R, Redon C, Solier S, Agama K, Huang D, Hollingshead M, Rubinstein L, Chen A, Kummar S, Parchment R, Tomaszewski J, Pommier Y, Bonner W, Doroshow J. A novel immunoassay (ELISA) for quantitative γH2AX detection and pharmacodynamic monitoring of DNA damage induced by chemotherapeutic agents and PARP inhibitors. 2011. AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics Abstract #A46.
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4. Ji J, Zhang Y, Redon CE, Reinhold WC, Chen AP, Fogli LK, Holbeck SL, Parchment RE, Hollingshead M, Tomaszewski JE, Dudon Q, Pommier Y, Doroshow JH, Bonner WM. Phosphorylated fraction of H2AX as a measurement for DNA damage in cancer cells and potential applications of a novel assay. (2017) PLoS One. 12(2); e0171582. PMID: 28158293.
5. SOP documents can be found on the DCTD Biomarkers website:
    - **Landing Page**: http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm
    - **SOP340520 LINK:** http://dctd.cancer.gov/ResearchResources/biomarkers/docs/par/SOP340520_Biopsy_Tissue.pdf
    - **SOP340506 LINK:** https://dctd.cancer.gov/ResearchResources/biomarkers/docs/par/SOP340506_PBMC_Extraction.pdf

APPENDIX 1: γH2AX ASSAY RECORD

NOTE: Record times using **military** time (24-h designation); for example, specify 16:15 to indicate 4:15 PM.

Assay Technician: _____

Date: _____

Plate ID (optional): _____

1. Key Reagents/Supplies for γH2AX Assay

The reagents listed below are considered key to the success of a reproducible assay. Tracking performance of lots used in this RUO protocol and qualifying new reagent lots against the previous lots (suggested performance criteria ±25% agreement) is recommended.

Reagent Name	Lot Number	Stock Concentration	Expiration Date
γH2AX Peptide Standard			
Tumor Lysate Controls: • High-C • Mid-C • Low-C			
γH2AX Mouse mAb			
H2AX Rabbit pAb			
Goat Anti-Rabbit HRP Conjugate			
Chemiluminescent Substrate: Pico Stable Peroxide and Luminol/Enhancer Solutions		N/A	
Reacti-Bind White Opaque 96-well Plate		N/A	N/A

ASSAY RECORD: INITIALS _____ DATE: _____

2. Preparation of Reagents for γH2AX Assay

A. Reagents

Buffers should be prepared based on volumes needed to complete all the steps with the number of 96-well plates in the experimental run. Always prepare at least 10% excess volume of buffer to ensure adequate volume to complete the study (scale-up appropriately for additional plates).

i. Coating Buffer: Dissolve one capsule of Carbonate-Bicarbonate Buffer in 50 mL of deionized water yielding 0.1 M Carbonate-Bicarbonate Buffer, pH 9.6 final. For each 96-well plate (prepare enough for 110 wells), 11 mL coating buffer will be needed. Keep at 2ºC to 8°C. Discard unused buffer at end of the experimental run.

ii. SuperBlock: For one 96-well plate (preparing for 110 wells), pipette 40 mL SuperBlock into a 50 mL tube. Keep at 2ºC to 8°C. Discard unused buffer at end of assay run.

iii. Wash Buffer (1X PBS-0.1% Tween): To prepare 1 L of buffer pipette 100 mL 10X PBS and 10 mL of 10% Tween 20 into 890 mL deionized water. Keep at 25°C ± 3°C for up to 1 wk.

iv. Assay Buffer/Diluent (1X PBS-2% BSA): To prepare 400 mL of assay diluent add 8 g BSA and 40 mL 10X PBS to 360 mL deionized water. Keep at 2ºC to 8°C for up to 2 wks.

v. γH2AX Sample Diluent (CEB Complete): To prepare 10 mL CEB Complete add 500 µL of 20% SDS, 100 µL of 100 mM PMSF, 400 µL of 25X PI, one tablet of PhosSTOP to 9.0 mL CEB. Keep at 2ºC to 8°C. Discard unused buffer at end of assay run.

3. Capture Antibody Preparation for γH2AX Assay:

A. Preparation of γH2AX mAb Coating Solution

Remove antibody from -20˚C freezer and place on ice.

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/ (1000 µL/mL) = 11 mL. Prepare **γH2AX mAb Coating Buffer** using the following calculations:

i. Dilution of γH2AX mAb **STOCK** = 1: _____

e.g., γH2AX mAb **STOCK** recommended dilution for Lot# DAM1567248 is 1:250.

$$\frac{11\text{ mL}}{\text{Recommended dilution of γH2AX mAb STOCK}} * 1000\ \mu\text{L/mL} = \underline{XX}\ \mu\text{L γH2AX mAb STOCK}$$

$$\frac{11\text{ mL}}{\frac{\quad}{\text{(dilution factor)}}} * 1000\ \mu\text{L/mL} = \_\_\_\_\_\ \mu\text{L γH2AX mAb STOCK}$$

ii. Place the following in a 15-mL polypropylene tube and mix by inversion 5 to 8 times.

11 mL	Coating Buffer
____ µL	γH2AX mAb **STOCK**

ASSAY RECORD: INITIALS _____ DATE: _____

B. Incubation Conditions for Coating Plate

Add 100 µL **γH2AX mAb Coating Solution** to each well, and incubate at 37°C for 2 h. Plates can also be coated overnight at 2°C to 8°C.

Date: _____ Start Time: ____ : ____ Incubation Temp: _____ °C

Date: _____ Stop Time: ____ : ____

4. Block Step for γH2AX Assay

After the capture antibody incubation, aspirate the wells and add 250 µL SuperBlock to each well and incubate at 37°C for 1 to 1.5 h (move to 25°C ± 3°C if blocking longer).

Incubation conditions for blocking plate:

Date: _____ Start Time: ____ : ____ Incubation Temp: _____ °C

Date: _____ Stop Time: ____ : ____

5. Preparation of Working Dilutions of Unknown Sample Lysates for γH2AX Assay

Normalize unknown lysates to 0.2 µg/µL working dilution prior to preparation of samples for the immunoassay.

Sample No.	**SampleID**	**Stock Lysate Conc.** xx µg/µL	**Working Lysate Conc.** 0.2 µg/µL	**Vol. Stock Lysate** (µL)	**Vol. CEB Complete** (85 µL - Vol. Stock Lysate used)
S1					
S2					
S3					
S4					
S5					
S6					
S7					
S8					
S9					
S10					

ASSAY RECORD: INITIALS _____ DATE: _____

6. Preparation of Unknown Samples (A) and γH2AX Peptide Standards (B) for γH2AX Assay

A. Unknown Sample Calculation Table

Unknown samples are run in triplicate, 25 µL sample/well (preparing 1 well extra). Sample numbers correspond to those on the Plate Map Design (Section 6.1). *It may be necessary to further dilute certain cell line lysates with high γH2AX in order to obtain a quantitative value in the range of the assay. In the event that a cell line provides values above the range of the assay (>ULQ), appropriately pre-dilute with CEB Complete and re-run.

All Samples		**Tumor/Tissue Samples**				**PBMC/Cell Samples**	
Sample No.	**Sample Name/ID**	**Working Lysate Conc.**	**Diluted Lysate** (2, 1, and 0.5 µg/well)			**Stock Cell Conc.**	**Stock Lysate Vol. (µL)**
		0.2 µg/µL	**Vol. Working Lysate (µL)**	**Vol. CEB Complete (**100 µL - Vol. Lysate**)**	**Final conc. (µg/well)**	*1 x 10^7 cells/mL	100 µL
S1		µg/µL			µg/well	cells/mL	
S2		µg/µL			µg/well	cells/mL	
S3		µg/µL			µg/well	cells/mL	
S4		µg/µL			µg/well	cells/mL	
S5		µg/µL			µg/well	cells/mL	
S6		µg/µL			µg/well	cells/mL	
S7		µg/µL			µg/well	cells/mL	
S8		µg/µL			µg/well	cells/mL	
S9		µg/µL			µg/well	cells/mL	
S10		µg/µL			µg/well	cells/mL	
S11		µg/µL			µg/well	cells/mL	
S12		µg/µL			µg/well	cells/mL	
S13		µg/µL			µg/well	cells/mL	
S14		µg/µL			µg/well	cells/mL	
S15		µg/µL			µg/well	cells/mL	
S16		µg/µL			µg/well	cells/mL	

B. γH2AX Peptide Standards

Calculations for preparation of the 1920 pM γH2AX standard in tube #1.

Supplied γH2AX peptide standard = _____ pM

e.g., γH2AX peptide standard **Stock** γH2AX JJ2, Lot# 113011 is supplied at 20680 pM (64 ng/ml).

$$\left(\frac{1920\ \text{pM}}{\text{Conc. of } \gamma\text{H2AX standard Stock (pM)}}\right) * 200\ \mu\text{L} = \underline{\text{XX}}\ \mu\text{L } \gamma\text{H2AX standard Stock solution in 200 µL final}$$

$$\left(\frac{1920\ \text{pM}}{\_\_\_\_\_\ (\text{pM})}\right) * 200\ \mu\text{L} = \_\_\_\_\_\ \mu\text{L } \gamma\text{H2AX standard Stock solution in } \mathbf{\underline{200\ \mu L}} \text{ final}$$

Serial dilutions of the γH2AX peptide standards are used to prepare the remaining tubes with concentrations ranging from 960 to 15 pM in Assay Buffer (1X PBS-2% BSA). 25 µL of each diluted standard will be added to the standard wells in the 96-well plate containing 50 µL of Assay Buffer (1X PBS-2% BSA) to give a 3-fold dilution which generates a reference standard curve ranging from 640 to 5 pM γH2AX standards. Label tubes with final concentration of standard.

Tube # (Plate Row)	Vol. and Source of Concentrated Standard	Vol. 1X PBS-2% BSA	Resulting Conc. of Diluted Standard	Conc. of Standard in Plate (1:3 Dilution)
1 (H)	_____ µL of γH2AX Standard Stock Solution	_____ µL (bring to 200 µL)	1920 pM	640 pM
2 (G)	100 µL of tube #1	100 µL	960 pM	320 pM
3 (F)	100 µL of tube #2	100 µL	480 pM	160 pM
4 (E)	100 µL of tube #3	100 µL	240 pM	80 pM
5 (D)	100 µL of tube #4	100 µL	120 pM	40 pM
6 (C)	100 µL of tube #5	100 µL	60 pM	20 pM
7 (B)	100 µL of tube #6	100 µL	30 pM	10 pM
8 (A)	100 µL of tube #7	100 µL	15 pM	5 pM
9 (Blank)	0 µL	600 µL	0 pM	0 pM (0 pM)

C. Controls (see Appendix 3 for preparation)

The High-, Mid- and Low-C tumor lysate controls for the γH2AX assay are prepared from 1 x 10^7 cells/mL stock lysates of irradiated MCF7 and untreated MCF7 cultured tumor cell lines. Controls will be diluted an additional 3-fold when added to the 96-well plate.

High-C: irradiated MCF7 lysate at 1 x 10^7 cells/mL.

Mid-C: mix 30% High-C and 70% Low-C.

Low-C: untreated MCF7 lysate 1 x 10^7 cells/mL.

Controls are aliquoted for single use, thaw only what is needed for the run.

Note the Control Numbers below:

High-C: _____

Mid-C: _____

Low-C: _____

7. Plate Incubation for γH2AX Assay

Add 25 µL unknown samples, tumor controls, and γH2AX peptide standards to the 96-well plate (wells contain 50 µL 1X PBS-2% BSA), cover plate, and incubate at 2°C to 8°C for 18 ± 2 h.

Date: _____ Start Time: ____ : ____ Incubation Temp: _____ °C

Date: _____ Stop Time: ____ : ____

8. Detection Antibody: H2AX Rabbit pAb

A. Preparation of H2AX Rabbit pAb Working Solution (100 µL/well)

Remove antibody from -20˚C freezer and place on ice.

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/ (1000 µL/mL) = 11 mL. Prepare **H2AX Rabbit pAb Working Solution** using the following calculations:

i. Dilution of H2AX Rabbit pAb **STOCK** = 1: _____

ii. e.g., H2AX pAb **STOCK** recommended dilution for Lot# 778559 is 1:850

$$\frac{11\ \text{mL}}{\text{Recommended dilution of H2AX Rabbit pAb } \textbf{STOCK}} * 1000\ \mu\text{L/mL} = \underline{XX}\ \mu\text{L H2AX Rabbit pAb } \textbf{STOCK}$$

$$\frac{11\ \text{mL}}{\text{(dilution factor)}} * 1000\ \mu\text{L/mL} = \_\_\_\_\ \mu\text{L H2AX Rabbit pAb } \textbf{STOCK}$$

iii. Place the following in a 15-mL polypropylene tube:

11 mL	1X PBS-2% BSA
11 µL	Mouse serum (1:1000) Lot #: _____
____ µL	H2AX Rabbit pAb **STOCK**

iv. Mix by inversion 5 to 8 times, and let stand at 25°C ± 3°C for 15 min before use.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

B. Addition of Prepared H2AX Rabbit pAb Working Solution

Add 100 µL of the H2AX **Rabbit pAb Working Solution** to each well and incubate for 2 to 2.5 h at 25°C ± 3°C.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

9. Reporter: HRP Conjugate for γH2AX Assay

A. Preparation of HRP Conjugate Working Solution (100 µL/well)

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/ (1000 µL/mL) = 11 mL. Prepare **HRP Conjugate Working Solution** using the following calculations:

i. Recommended dilution of Goat Anti-Rabbit HRP Conjugate **STOCK** = 1: _____

ii. e.g., HRP Conjugate **STOCK** recommended dilution for Lot# 101008 is 1:1000

$$\frac{11\text{ mL}}{\text{Recommended dilution of HRP Conjugate STOCK}} * 1000\ \mu\text{L/mL} = \underline{XX}\ \mu\text{L HRP Conjugate STOCK}$$

$$\frac{11\text{ mL}}{\text{(dilution factor)}} * 1000\ \mu\text{L/mL} = \underline{\quad}\ \mu\text{L HRP Conjugate STOCK}$$

iii. Place the following in a 15-mL polypropylene tube:

11 mL	1X PBS-2% BSA
11 µL	Mouse serum (1:1000) Lot #: _____
___ µL	HRP Conjugate **STOCK**

iv. Mix by inversion 5 to 8 times, and let stand at 25°C ± 3°C for 15 min before use.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

B. Addition of HRP Conjugate Working Solution

Add 100 µL of the **HRP Conjugate Working Solution** to each of the washed wells and incubate in the dark for 1 to 1.5 h at 25°C ± 3°C.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

10. Chemiluminescent Substrate for γH2AX Assay

A. Preparation of Substrate Solution (100 µL/well)

Calculate volume of substrate required for the experimental run. For one 96-well plate, prepare 110 wells: (100 µL/well*110)/ (1000 µL/mL) = 11 mL. Immediately before washing the plate, prepare the following in a 15-mL polypropylene tube wrapped with aluminum foil. Mix by inversion 5 to 8 times and keep at 25°C ± 3°C in the dark until use.

5.5 mL Pico Stable Peroxide	(50 µL/well*110)/ (1000 µL/mL)
5.5 mL Pico Luminol/Enhancer	(50 µL/well*110)/ (1000 µL/mL)

Time of Substrate Preparation: _____ : _____

B. Substrate Solution Incubation and RLU Reading Times

Time of Substrate Addition to Wells: _____ : _____

Time Initial RLU Reading is Captured: _____ : _____

Time Final RLU Reading is Captured (opt): _____ : _____

11. Notes, including any deviations from the SOP:

APPENDIX 2: TOTAL H2AX ASSAY RECORD

NOTE: Record times using **military** time (24-h designation); for example, specify 16:15 to indicate 4:15 PM.

Assay Technician: _____

Date: _____

Plate ID (optional): _____

1. Key Reagents/Supplies for Total H2AX Assay

The reagents listed below are considered key to the success of a reproducible assay. Tracking performance of lots used in this RUO protocol and qualifying new reagent lots against the previous lots (suggested performance criteria ±25% agreement) is recommended.

Reagent Name	Lot Number	Stock Concentration	Expiration Date
Total H2AX Recombinant Standard			
Tumor Lysate Control: High-C Mid-C Low-C			
Total H2AX Mouse mAb			
H2AX Rabbit pAb			
Goat Anti-Rabbit HRP Conjugate			
Chemiluminescent Substrate: Pico Stable Peroxide and Luminol/Enhancer Solutions		N/A	
Reacti-Bind White Opaque 96-well Plate		N/A	N/A

ASSAY RECORD: INITIALS _____ DATE: _____

2. Preparation of Reagents for Total H2AX Assay

A. Reagents

Buffers should be prepared based on volumes needed to complete all the steps with the number of 96-well plates in the experimental run. Always prepare at least 10% excess volume of buffer to ensure adequate volume to complete the study (scale-up appropriately for additional plates).

i. Coating Buffer: Dissolve one capsule of Carbonate-Bicarbonate Buffer in 50 mL of deionized water yielding 0.1 M Carbonate-Bicarbonate Buffer, pH 9.6 final. For each 96-well plate (prepare enough for 110 wells), 11 mL coating buffer will be needed. Keep at 2ºC to 8°C. Discard unused buffer at end of the experimental run.

ii. SuperBlock: For one 96-well plate (preparing for 110 wells), pipette 40 mL SuperBlock into a 50-mL tube. Keep at 2ºC to 8°C. Discard unused buffer at end of assay run.

iii. Wash Buffer - 1X PBS- 0.1% Tween: To prepare 1 L of buffer pipette 100 mL 10X PBS and 10 mL of 10% Tween 20 into 890 mL deionized water. Keep at 25°C ± 3°C for up to 1 wk.

iv. Assay Buffer - 1X PBS-2% BSA: To prepare 400 mL of buffer add 8 g BSA and 40 mL 10X PBS to 360 mL deionized water. Keep at 2ºC to 8°C for up to 2 wks.

v. Sample Diluent Buffer–CEB Complete: To prepare 10 mL CEB Complete add 500 µL of 20% SDS, 100 µL of 100 mM PMSF, 400 µL of 25X PI, one tablet of PhosSTOP to 9.0 mL CEB. Keep at 2ºC to 8°C. Discard unused buffer at end of assay run.

vi. Assay Buffer (1X PBS-2% BSA) containing 2 µg/mL (100 ng/well) of Protamine: To prepare 6 mL of buffer add 20 µL of 600 µg/mL Protamine working stock and 5980 µL of 1X PBS-2% BSA. Keep at 2ºC to 8°C. The final concentration used in the assay is 100 ng/well. Discard unused buffer at end of the experimental run.

Note: To make 600 µg/mL Protamine stock: dilute 120 mg of Protamine powder in 2 mL of dH_2O, this gives 60 mg/mL stock. Make two additional 10X serial dilution (1:100 dilution) with dH_2O to generate 600 µg/mL working stock.

3. Capture Antibody: Total H2AX mAb

A. Preparation of Total H2AX mAb Coating Solution

Remove antibody from -20˚C freezer and place on ice.

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/ (1000 µL/mL) = 11 mL. Prepare **Total H2AX mAb Coating Solution** using the following calculations:

i. Dilution of Total H2AX mAb **STOCK** = 1: _____

e.g., Total H2AX mAb **STOCK** recommended dilution for Lot# 09281-4F10 (1mg/mL) is 1:250.

$$\frac{11\text{ mL}}{\text{Recommended dilution of Total H2AX mAb STOCK}} * 1000\ \mu\text{L/mL} = \underline{XX}\ \mu\text{L Total H2AX mAb STOCK}$$

$$\frac{11\text{ mL}}{\text{(dilution factor)}} * 1000\ \mu\text{L/mL} = \_\_\_\_\_\ \mu\text{L Total H2AX mAb STOCK}$$

ii. Place the following in a 15-mL polypropylene tube and mix by inversion 5 to 8 times.

11 mL	Coating Buffer
____ µL	Total H2AX mAb **STOCK**

B. Incubation Conditions for Coating Plate

Add 100 µL **Total H2AX mAb Coating Solution** to each well, and incubate at 37°C for 2 h. Plates can also be coated overnight at 2°C to 8°C.

Date: _____ Start Time: ____:____ Incubation Temp: _____°C

Date: _____ Stop Time: ____:____

4. Block Step for Total H2AX Assay

After the capture antibody incubation, aspirate wells, and place 250 µL SuperBlock in each well and incubate at 37°C for 1 to 1.5 h (move to 25°C ± 3°C if blocking longer).

Incubation conditions for blocking plate:

Date: _____ Start Time: ____:____ Incubation Temp: _____°C

Date: _____ Stop Time: ____:____

5. Preparation of Working Dilutions of Unknown Tumor/Tissue Lysates for Total H2AX Assay

Normalize unknown biopsy lysates to 0.75 µg/µL working dilution with CEB Complete if the unknown stock lysate concentrations are **≥ 0.75 µg/µL** prior to preparation of samples for the immunoassay. Then dilute the 0.75 µg/µL normalized working lysate or 0.2 to 0.75 µg/µL stock lysates to 0.1 µg/µL with 1X PBS-2% BSA.

Sample No.	**Sample ID**	**Stock Lysate Conc.**	**Prediluted Lysate Conc. (Only for ≥ 0.75 µg/µL)**	**Vol. Stock Lysate (Only for for ≥ 0.75 µg/µL)**	**Vol. CEB Complete (Only for ≥ 0.75 µg/µL)**	**Vol. 0.2 µg/µL to 0.75 µg/mL Stock or Prediluted Lysate**	**Diluted Working Lysate Conc.**	**Vol. 1X PBS-2% BSA**
		xx µg/µL	0.75 µg/µL or N/A	(µL)	(10 µL - Vol. Stock Lysate)	(10 µL or XX µL)	0.1 µg/µL	(75 µL - Vol. 0.1 µg/µL Lysate)
S1								
S2								
S3								
S4								
S5								
S6								
S7								
S8								
S9								
S10								

ASSAY RECORD: INITIALS _____ DATE: _____

6. Preparation of Unknown Samples (A), Total H2AX Recombinant Standards (B) for Total H2AX Assay

A. Unknown Sample Calculation Table:

Unknown samples are run in triplicate, 25 µL sample/well (preparing 1 well extra). Sample numbers correspond to those on the Plate Map.

All Samples		**Tumor/Tissue Samples**				**PBMC/Cell Samples**		
Sample No.	**Sample ID**	**Working Lysate Conc.**	**Diluted Lysate** (0.5, 0.375 and 0.25 µg/well)			**Stock Cell Conc.**	**Stock Lysate Vol. (µL)**	**Vol. 1X PBS-2% BSA (µL)**
		0.1 µg/µL	**Vol. Working Lysate (µL)**	**Vol. 1X PBS-2% BSA (**100 µL - Vol. Lysate**)**	**Final conc. (µg/well)**	1 x 10^7 cells/mL	7.5 µL	117.5 µL
S1		µg/µL			µg/well	cells/mL		
S2		µg/µL			µg/well	cells/mL		
S3		µg/µL			µg/well	cells/mL		
S4		µg/µL			µg/well	cells/mL		
S5		µg/µL			µg/well	cells/mL		
S6		µg/µL			µg/well	cells/mL		
S7		µg/µL			µg/well	cells/mL		
S8		µg/µL			µg/well	cells/mL		
S9		µg/µL			µg/well	cells/mL		
S10		µg/µL			µg/well	cells/mL		
S11		µg/µL			µg/well	cells/mL		
S12		µg/µL			µg/well	cells/mL		
S13		µg/µL			µg/well	cells/mL		
S14		µg/µL			µg/well	cells/mL		
S15		µg/µL			µg/well	cells/mL		
S16		µg/µL			µg/well	cells/mL		

ASSAY RECORD: INITIALS _____ DATE: _____

B. Total H2AX Recombinant Protein Standards

Calculations for preparation of the 19200 pM Total H2AX standard in tube #1.

Supplied Total H2AX recombinant protein standard = _____ pM

e.g., Total H2AX standard **Stock** Lot# L16677 is supplied at 33000 pM (500 ng/ml)

$$\left(\frac{19200\ \text{pM}}{\text{Conc, of Total H2AX standard Stock (pM)}}\right) * 200\ \mu\text{L} = \underline{\text{XX}}\ \mu\text{L Total H2AX standard Stock solution in 200 µL final}$$

$$\left(\frac{19200\ \text{pM}}{\_\_\_\_\_\ (\text{pM})}\right) * 200\ \mu\text{L} = \_\_\_\_\ \mu\text{L total H2AX standard Stock solution in } \underline{\textbf{200 µL}} \text{ final}$$

Serial dilutions of the Total H2AX recombinant standards are used to prepare the remaining tubes with concentrations ranging from 9600 to 150 pM in 1X PBS-2% BSA. 25 µL of each diluted standard will be added to the standard wells in the 96-well plate containing 50 µL of Assay Buffer (1X PBS-2% BSA) with 2 µg/mL Protamine (100 ng/well of Protamine at 50 µL) which equals a 3-fold dilution of standards to generate a reference standard curve ranging from 6400 to 50 pM Total H2AX standards. Label tubes with final concentration of standard.

Tube # (Plate Row)	Vol. and Source of Concentrated Standard	Vol. 1X PBS-2% BSA	Resulting Conc. of Diluted Standard	Conc. of Standard in Plate (1:3 Dilution)
1 (H)	_____ µL of Stock Solution	_____ µL	19200 pM	6400 pM
2 (G)	100 µL of tube #1	100 µL	9600 pM	3200 pM
3 (F)	100 µL of tube #2	100 µL	4800 pM	1600 pM
4 (E)	100 µL of tube #3	100 µL	2400 pM	800 pM
5 (D)	100 µL of tube #4	100 µL	1200 pM	400 pM
6 (C)	100 µL of tube #5	100 µL	600 pM	200 pM
7 (B)	100 µL of tube #6	100 µL	300 pM	100 pM
8 (A)	100 µL of tube #7	100 µL	150 pM	50 pM
9 (Blank)	0 µL	600 µL	0 pM	0 pM

C. Controls (see Appendix 3 for preparation)

Controls for the Total H2AX are prepared as specified in the table below and are aliquoted for single use. Thaw control amounts as needed for the run.

Control Tube Dilution	**Vol. and Source of Control Lysate**	**Vol.** *1X PBS-2% BSA*	**Conc. of Control in Plate (1:3 Dilution)**
High-C (1:16.7) *1 x 10^7 cells/mL stock lysate	**7.5** µL of SN12 C Tumor Cell Stock	117.5µL	High-C (1:50)
Mid-C (1:16.7) *1 x 10^7 cells/mL stock lysate	**9** µL of MCF7 Tumor Cell Stock	141 µL	Mid-C (1:50)
Low-C (1:66.7)	**30** µL of Mid-C (MCF7) (1:50)	90 µL	Low-C (1:200)

Note the Control Lot Numbers below:

High-C: _____

Mid-C: _____

Low-C: _____

7. Plate Incubation for Total H2AX Assay

Add 25 µL unknown samples, tumor controls, and Total H2AX standards to the 96-well plate to wells containing 50 µL 1X PBS-2% BSA - 2 µg/mL Protamine (100 ng/well of Protamine), cover plate, and incubate at 2°C to 8°C for 18 ± 2 h.

Date: _____ Start Time: ____ : ____ Incubation Temp: _____ °C

Date: _____ Stop Time: ____ : ____

8. Detection Antibody: H2AX Rabbit pAb

A. Preparation of H2AX Rabbit pAb Working Solution (100 µL/well)

Remove antibody from -20˚C freezer and thaw on ice.

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/ (1000 µL/mL) = 11 mL. Prepare **H2AX Rabbit pAb Working Solution** using the following calculations:

i. Dilution of H2AX Rabbit pAb **STOCK** = 1: _____

ii. e.g., total H2AX pAb **STOCK** recommended dilution for Lot# 778559 is 1:850.

$$\frac{11\ \text{mL}}{\text{Recommended dilution of H2AX Rabbit pAb } \textbf{STOCK}} * 1000\ \mu\text{L/mL} = \underline{\text{XX}}\ \mu\text{L H2AX Rabbit pAb } \textbf{STOCK}$$

$$\frac{11\ \text{mL}}{\text{(dilution factor)}} * 1000\ \mu\text{L/mL} = \_\_\_\_\ \mu\text{L H2AX Rabbit pAb } \textbf{STOCK}$$

iii. Place the following in a 15-mL polypropylene tube:

11 mL	1X PBS-2% BSA
11 µL	Mouse serum (1:1000) Lot #: _____
____ µL	H2AX Rabbit pAb **STOCK**

iv. Mix by inversion 5 to 8 times, and let stand at 25°C ± 3°C for 15 min before use.

Start Time: ____ : ____ Stop Time: ____ : ____ Incubation Temp: _____ °C

B. Addition of Prepared H2AX Rabbit pAb Working Solution

Add 100 µL of the H2AX **Rabbit pAb Working Solution** to each well and incubate for 2 to 2.5 h at 25°C ± 3°C.

Start Time: ____ : ____ Stop Time: ____ : ____ Incubation Temp: _____ °C

DATA RECORD: INITIALS _____ DATE: _____

9. Reporter: HRP Conjugate for Total H2AX Assay

A. Preparation of HRP Conjugate Working Solution (100 µL/well)

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/ (1000 µL/mL) = 11 mL. Prepare **HRP Conjugate Working Solution** using the following calculations:

i. Recommended dilution of Goat Anti-Rabbit HRP Conjugate **STOCK** = 1: _____

ii. e.g., HRP Conjugate **STOCK** recommended dilution for Lot# 120504 is 1:1000

$$\frac{11\text{ mL}}{\text{Recommended dilution of HRP Conjugate STOCK}} * 1000\ \mu\text{L/mL} = \underline{XX}\ \mu\text{L HRP Conjugate STOCK}$$

$$\frac{11\text{ mL}}{\text{(dilution factor)}} * 1000\ \mu\text{L/mL} = \_\_\_\_\_\ \mu\text{L HRP Conjugate STOCK}$$

iii. Place the following in a 15-mL polypropylene tube:

11 mL	1X PBS-2% BSA
11 µL	Mouse serum (1:1000) Lot #: _____
___ µL	HRP Conjugate **STOCK**

iv. Mix by inversion 5 to 8 times, and let stand at 25°C ± 3°C for 15 min before use.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

B. Addition of HRP Conjugate Working Solution

Add 100 µL of the **HRP Conjugate Working Solution** to each of the washed wells and incubate in the dark for 1 to 1.5 h at 25°C ± 3°C.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

10. Chemiluminescent Substrate Total H2AX Assay

A. Preparation of Substrate Solution (100 µL/well)

Calculate volume of substrate required for the experimental run. For one 96-well plate, prepare 110 wells: (100 µL/well*110)/ (1000 µL/mL) = 11 mL. Immediately before washing the plate, prepare the following in a 15-mL polypropylene tube wrapped with aluminum foil. Mix by inversion 5 to 8 times and keep at 25°C ± 3°C in the dark until use.

5.5 mL	Pico Stable Peroxide	(50 µL/well*110)/ (1000 µL/mL)
5.5 mL	Pico Luminol/Enhancer	(50 µL/well*110)/ (1000 µL/mL)

Time of Substrate Preparation: _____ : _____

B. Substrate Solution Incubation and RLU Reading Times

Time of Substrate Addition to Wells: _____ : _____

Time Initial RLU Reading is Captured: _____ : _____

Time Final RLU Reading is Captured (opt): _____ : _____

11. Notes, including any deviations from the SOP:

APPENDIX 3: PREPARATION OF TUMOR LYSATE CONTROL SAMPLES

1.0 MATERIALS AND REAGENTS

1.1 Pipettors (200-1000 µL) and tips
1.2 Automatic pipettor
1.3 Vacuum filter/storage bottle system, 0.22-µm pore, 500 mL (e.g., Corning, Cat#: 430758).
1.4 1-, 5-, 10-, and 25-mL pipettes, sterile, individually wrapped (e.g., Fisher Scientific, Cat#: 13-675-15C, 13-675-22, 13-675-20, and 13-668-2)
1.5 1.5-mL Sarstedt tubes (e.g., Sarstedt, Cat#: 72.692.005)
1.6 15-mL polypropylene tubes (e.g., Fisher Scientific, Cat#: 14-959-49B or Becton Dickinson, Cat#: 352097)
1.7 50-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352098)
1.8 Cell culture flask, 75 cm^2, vent cap (T75; e.g., Corning, Cat#: 3290)
1.9 Ice bucket
1.10 100% Ethanol
1.11 MCF7 human breast adenocarcinoma (ATCC, Cat#: HTB-22)
1.12 SN12C human renal cancer cell (NCI)
1.13 10X phosphate buffered saline (PBS; e.g., Invitrogen, Cat#: 70013-073) [Dilute 1:10 in DI water to prepare 1X PBS for use in assay]
1.14 RPMI-1640 medium, 500-mL bottles (e.g., Invitrogen, Cat#: 22400089)
1.15 L-Glutamine, 200 mM (e.g., Invitrogen, Cat#: 25030164)
1.16 Fetal bovine serum, 500-mL bottles (FBS; Gemini Bio-Products, Cat#: 100-106) [Store at -20°C as 50-mL aliquots in 50-mL polypropylene tubes]
1.17 Trypsin Solution, 0.25%, 1X with EDTA (e.g., Invitrogen, Cat#: 25200056)
1.18 Cell Extraction Buffer (CEB; Invitrogen, Cat#: FNN0011)
1.19 20% sodium dodecyl sulfate (SDS; e.g., Sigma-Aldrich, Cat#: 05030-500ML-F)
1.20 Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, Cat#: 93482-50ML-F)
1.21 Protease Inhibitor (PI) Cocktail (Sigma-Aldrich, Cat#: P-2714 or Roche, Cat#: 11 697 498 001)
1.22 PhosSTOP, phosphatase inhibitor cocktail tablets (Roche, Cat#: 04906837001)
1.23 Liquid nitrogen or dry ice/ethanol bath
1.24 Hemocytometer and cover slips or an automated cell counter (e.g., Vi-Cell)
1.25 100°C heat block or boiling water bath
1.26 Vortex Genie 2 (Daigger, Cat#: 3030A)
1.27 Table-top centrifuge with a swing-bucket rotor, refrigerated (e.g., Sorvall Legend RT centrifuge (Fisher Scientific) with a Swing Bucket Rotor (Fisher Scientific, Cat#: 75-006-434) and manufacturer-recommended tube adaptors
1.28 Sorvall Fresco microcentrifuge (Fisher Scientific)
1.29 Class II Type A2 biosafety cabinet/tissue culture laminar flow hood
1.30 37°C tissue culture incubator, humidified, 5% CO_2,
1.31 37°C water bath
1.32 -20°C and -80°C freezers

2.0 PROTOCOL

2.1 Prepare Tissue Culture Medium

Note: Growth medium can be prepared ahead of time and stored at 4°C for up to 3 weeks.

2.1.1 Using sterile technique, prepare complete RPMI-1640 growth medium (RPMI-1640 + 10% FBS + 1% L-glutamine).

2.1.2 For each 500-mL bottle of RPMI-1640 to be prepared, thaw a 50-mL aliquot of FBS and a 5-mL aliquot of 200 mM L-glutamine in a 37°C water bath and then move to a laminar flow hood. Clean the outside of tubes with 70% ethanol.

2.1.3 Using a disposable filter unit and sterile technique, filter 445 mL RPMI-1640, 50 mL FBS, and 5 mL of 200 mM L-glutamine.

2.1.4 Label bottle with lot number and expiration date of medium components (if applicable) and date of preparation. Store at 4°C until use.

2.2 Thawing Frozen MCF7 or SN12C Cells

2.2.1 Pre-warm growth medium in a 37°C water bath for 15 to 20 min.

2.2.2 Frozen cell stocks (~1 mL) should be quickly thawed in a 37°C water bath until just a little bit of ice remains. Immediately transfer the vial to a laminar flow hood, wipe the exterior with ethanol, and transfer into a 15 mL polypropylene tube containing 9 mL growth medium.

2.2.3 Gently pellet cells by centrifugation at 200 x *g* in a swing-bucket rotor at 4°C for 10 min. Aspirate and discard the supernatant.

2.2.4 Gently resuspend the cell pellet in 10 mL growth medium and transfer into a T75 flask containing an additional 15 mL growth medium (25 mL total volume).

2.2.5 Label the flask with the date and passage number of the cells and place in 37°C, 5% CO_2, humidified incubator.

2.2.6 Cultured cells should become confluent with 4 to 5 days.

2.3 Subculture MCF7 or SN12C Cells

Note: Cells maintained in tissue culture should be regularly split to maintain overall cell line health. Cells should be cultured for no longer than 30 passages before preparing a new culture with a fresh vial of cells.

2.3.1 Pre-warm growth medium in a 37°C water bath and trypsin at RT for 15 to 20 min.

2.3.2 In a laminar flow hood, aspirate old medium and add 5 mL RT trypsin solution. Gently rock the container to get complete coverage of the cells. Leave at RT for 10 to 30 sec.

2.3.3 Carefully, aspirate and discard the trypsin without disturbing the cells and place flask in a 37°C, 5% CO_2, humidified incubator for 30 to 60 sec.

2.3.4 Tap flask by hand to release cells from the bottom and then add 10 mL of growth medium to the flask. Pipette up and down 2 to 3 times to disperse cell clumps.

2.3.5 Prepare 5 new T75 flasks to split the current MCF7 cell culture by adding 23 mL growth medium to each flask and labeling the flask with the date and the new passage number for the cells. Add 2 mL of cell suspension from previous step into each flask.

2.3.6 Incubate flasks in a 37°C, 5% CO_2, humidified incubator. Cells should become confluent with 4 to 5 days.

2.3.7 For γH2AX induction to create the High γH2AX Control (High-C), MCF-7 cells are irradiated at 10 Gy and returned to the 37°C, 5% CO_2 incubator for 30 min prior to harvest.

2.4 Harvest Cultured MCF7 or SN12C Cells

2.4.1 On the day of cell harvest for preparation of the Tumor Cell Control Lysates for SOP340024, five T75 flasks of cells should be 80% to 90% confluent.

2.4.2 Pre-warm growth medium in a 37°C water bath and trypsin and 1X PBS at RT for 15 to 20 min.

2.4.3 In a laminar flow hood, aspirate old medium from all five T75 flasks and wash the cell monolayer surface once with 10 mL RT 1X PBS.

2.4.4 Aspirate PBS, add 5 mL RT trypsin to each flask. Gently rock the container to get complete coverage of the cells. Leave at RT for 2 to 3 min.

2.4.5 Carefully, aspirate and discard the trypsin without disturbing the cells and let flask incubate for an additional 2 to 3 min at RT. Observe the cells, the monolayer will begin to detach from flask bottom.

2.4.6 Add 10 mL RT 1X PBS to each flask and pipette up and down several times to disperse cell clumps. Transfer the cells from each flask into 15-mL polypropylene tubes.

2.4.7 Gently pellet cells by centrifugation at 200 x *g* in a swing-bucket rotor at 4°C for 10 min. Aspirate and discard the supernatant.

2.4.8 Wash the cell pellets with 10 mL 1X RT PBS and pipette up and down several times to disperse cell clumps. Pool all MCF7 or SN 12C cell suspensions into a 50-mL polypropylene tube, respectively, so cells are evenly dispersed.

2.4.9 Using either a hemocytometer or automatic cell counter (e.g., Vi-Cell) determine the total viable cell count in the pooled cell suspension.

- MCF7 or SN12C cells must be ≥ 90% viable to be used for control lysate preparation. If the cells are < 90% viable, they should be discarded and a fresh culture of cells will need to be used for control lysate preparation.

2.4.10 Adjust the total viable cell concentration to 3 x 10^6 cells/mL and then aliquot 1 mL volumes into 1.5 mL Sarstedt tubes.

2.4.11 Centrifuge aliquots at 12,000 x *g* in a Sorvall microcentrifuge at 4°C for 5 min. Aspirate and discard the supernatant and place tubes on ice.

2.4.12 If not used immediately, snap-freeze the cell pellets in liquid nitrogen or a dry ice/ethanol bath. Store the frozen cell pellets in a freezer box at -80°C.

2.5 MCF7 or SN12C Cell Lysis

2.5.1 Add 300 µL Cell Extraction Buffer (CEB) containing 1X protease inhibitor (PI) cocktail and 1 mM PMSF per 3 x 10^6 cells to fresh or frozen cell pellets, this should yield a relative cell concentration of ~1 x 10^7 cells/mL.

2.5.2 Vortex tube for 3 to 5 sec at medium speed (setting 5-6 on Vortex Genie 2). Ensure the cell pellet is dislodged and mixing gently in the CEB.

2.5.3 Place tube on ice and incubate the cells in the CEB for 30 min with 3 to 5 sec of vortexing at 10-min intervals.

2.5.4 Move samples to room temperature and add 20% SDS to a final concentration of 1% SDS (e.g., 15 µL 20% SDS to 300 µL lysate).

2.5.5 Vortex tube for 3 to 5 sec at medium speed to distribute the SDS in the buffer.

2.5.6 Boil the cell extract for 5 min in a 100°C heat block or boiling water bath, and cool down on ice.

2.5.7 Clarify the extract by centrifugation in a Sorvall Fresco microcentrifuge at 12,000 x g for 10 min at 4°C. Transfer supernatant into a new Sarstedt tube and hold on ice. Discard the original tube with any precipitated material in the appropriate waste container.

2.5.8 Label each Sarstedt tube with a Lot Identification Code (date of cell harvesting, lysis, and freezing).

2.5.9 If not used immediately for the immunoassay, snap-freeze the protein extract in liquid nitrogen or a dry ice/ethanol bath. Store the frozen lysates in a freezer box at -80°C.

2.5.10 Designation of High, Mid, and Low Controls

2.6 γH2AX and H2AX Assay Controls

Note: NCI has used the following strategy for creation of the controls but each laboratory should qualify their own batch of controls.

2.6.1 γH2AX Assay Controls

2.6.1.1 The High-, Mid- and Low-C tumor lysate controls are prepared from a 1 x 10^7 cells/mL stock lysates of irradiated MCF7 and untreated MCF7 cultured tumor cell lines. Controls will be diluted an additional 3-fold when added to the 96-well plate.

- High-C: irradiated MCF7 lysate at 1 x 10^7 cells/mL.
- Mid-C: mix 30% High-C and 70% Low-C.
- Low-C: untreated MCF7 lysate 1 x 10^7 cells/mL.

2.6.2 Total H2AX Assay Controls

2.6.2.1 The High-, Mid- and Low-C tumor lysate controls are dilutions prepared from a 1 x 10^7 cells/mL stock lysates of SN12C and MCF7 cultured tumor cell lines. Controls will be diluted an additional 3-fold when added to the 96-well plate.

2.6.2.2 SN12C lysate (~1 x: 10^7 cells/mL) diluted at 1:50 will be used as High-C. MCF7 lysate (~1 x 10^7 cells/mL) diluted at 1:50 will be used as MID-C and MCF7 lysate diluted at 1:200 will be used as Low-C. A representative dilution scheme is shown in the table below.

Control Tube Dilution	**Vol. and Source of Control Lysate**	**Vol.** *1X PBS-2% BSA*	**Conc. of Control in Plate (1:3 Dilution)**
High-C (1:16.7) *1 x 10^7 cells/mL stock lysate	**7.5** µL of SN12 C Tumor Cell Stock	117.5µL	High-C (1:50)
Mid-C (1:16.7) *1 x 10^7 cells/mL stock lysate	**9** µL of MCF7 Tumor Cell Stock	141 µL	Mid-C (1:50)
Low-C (1:66.7)	**30** µL of Mid-C (MCF7) (1:50)	90 µL	Low-C (1:200)

2.6.3 New lots of tumor control lysates should be assayed in parallel with previously qualified controls using qualified assay reagents. The new tumor control lysates should provide control readouts with ±25% agreement to the previous lot. Use of the Westgard Multirule System and a control grid are recommended to identify changes in performance of the control samples across assay runs within a given laboratory. Controls are aliquoted for single use at 120-125 µL per vial.

2.6.4 Representative assay readout ranges for the γH2AX and Total H2AX Control Lysates are outlined in the Steps 4.3 and 4.4.

APPENDIX 4: ABBREVIATIONS

BSA	=	Bovine Serum Albumin
C	=	Control
Cat#	=	Catalog Number
CEB	=	Cell Extraction Buffer
CV	=	Coefficient of Variation
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
H	=	Hour
γH2AX	=	Histone H2AX Phosphorylated at Serine 139 (designed as Gamma)
HRP	=	Horse Radish Peroxidase
IA	=	Immunoassay
LHTP	=	Laboratory of Human Toxicology and Pharmacology
LLD	=	Lower Limit of Detection
LLQ	=	Lower Limit of Quantitation
LLQ-c	=	Lower Limit of Quantitative Concentration
min	=	Minute
mm	=	Milli-meter
MSec	=	Milli-second
mAb	=	Monoclonal Antibody
pAb	=	Polyclonal Antibody
PAR	=	Poly(ADP-ribose)
PBS	=	Phosphate Buffered Saline
PI	=	Protease Inhibitors
PMSF	=	Phenylmethanesulfonyl Fluoride
PD	=	Pharmacodynamic
RLU	=	Relative Light Units
RT	=	Room Temperature
RUO	=	Research Use Only
SD	=	Standard Deviation
SDS	=	Sodium Dodecyl Sulfate
SOP	=	Standard Operating Procedure
Temp	=	Temperature
UD	=	Undetectable
UQ	=	Unquantifiable
µL or µg	=	Microliter or Microgram
Y	=	Year

RUO PROTOCOL APPROVAL

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